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**The Expression and Role of the Extracellular Matrix
Protein Tenascin in Ovarian Cancer**

Katherine Elizabeth Wilson

**Doctor of Philosophy
Edinburgh University
1996**



For Mum, Dad and Nana

Declaration

In accordance with the regulations of the University, I declare that this thesis has been composed by myself entirely, and that the work presented is my own, except where acknowledgement has been indicated in the text.

Katherine Elizabeth Wilson

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Abstract

Tenascin (TN) is a large hexameric glycoprotein which is expressed transiently in the extracellular matrix at times of tissue development, proliferation and reorganisation. It is overexpressed in the stroma of several types of malignant tumours. The expression of TN in ovarian cancer has not previously been investigated. The aims of the project were therefore to (i) define the expression of TN in ovarian cancer, (ii) examine factors controlling its production and (iii) explore the functionality of the protein.

Initial studies characterised the expression of TN in a series of malignant, benign and borderline ovarian tumours. TN protein was examined by immunohistochemical staining of frozen sections of tumours. Malignant and borderline tumours showed a significantly greater incidence and intensity of stromal staining as compared to benign tumours. Omental metastases showed a pattern of protein distribution strikingly similar to their primary counterpart. These data suggest that TN is overexpressed in malignant ovarian tumours.

TN exists in a number of different isoforms, which are produced by alternative splicing of the RNA transcript. The pattern of different isoforms expressed was investigated using RT-PCR and hybridisation to specific oligonucleotides. The smallest TN splice variant was found in all tumours examined while the appearance of larger molecular weight transcripts was predominantly limited to malignant tumours.

In order to determine which cell types were capable of producing TN, *in vitro*, fibroblast cells, cultured from ascitic fluids of ovarian cancer patients, and established ovarian carcinoma cell lines were studied. TN levels in conditioned media were measured by ELISA. "Basal" levels of TN secretion were determined by culturing cells in serum-free media; under these conditions the ovarian fibroblasts secreted levels of TN over

100 fold greater than the epithelial cells. RT-PCR data showed that epithelial and fibroblast cell lines express TN RNA and display multiple RNA splice forms.

To examine whether a paracrine interaction between the fibroblast and epithelial cells can influence TN production, the cells were co-cultured in compartments separated by a filter, which allowed diffusion of soluble factors. The co-cultured populations of cells produced significantly more TN than either cell type alone. The effects of a number of potential modulating factors, on secretion of TN, have been investigated. Several factors (IGF II, TGF β , progesterone and EGF) stimulate TN secretion by fibroblasts while other factors (gonadotrophins and interferon) inhibit TN secretion in the same cell type. Of the factors studied TGF- β provided the greatest induction of TN in fibroblasts. None of these factors induced the PEO1 epithelial cell line to produce measurable levels of TN.

Adhesion and migration assays were used to examine how ovarian carcinoma cell lines interacted with TN, as compared with the ECM proteins fibronectin and collagen IV. The assays demonstrated that TN promoted cell adhesion, spreading and migration in the SKOV-3, 59M, PEO1 and PEO4 ovarian carcinoma cell lines, however, fibronectin and collagen IV appeared to be preferable substrates. Immuno-staining and analysis by flow cytometry of these cell lines demonstrated that all expressed the integrin $\alpha 2\beta 1$ which can bind TN, the SKOV-3 cell line also expressed the integrin $\alpha V\beta 3$.

These studies have demonstrated that TN is overexpressed in malignant ovarian tumours, and paracrine growth factors, such as TGF β , can induce the synthesis of TN in ovarian fibroblasts. Tumour cells can adhere to, and migrate on TN. These data would be consistent with TN playing a role in the invasion and metastasis of ovarian cancer

Abbreviations

ABC	- avidin biotin complex
BSA	- bovine serum albumin
Ci	- Curie
cpm	- counts per minute
DAB	- diaminobenzidine tetrahydrochloride
DMEM	- Dulbeccos modified eagle media
DMSO	- dimethyl sulphoxide
dNTP	- deoxynucleotide
ECM	- extracellular matrix
EDTA	- ethylenediaminetetraacetic acid
EGF	- epidermal growth factor
ELISA	- enzyme linked immunosorbant assay
FCS	- foetal calf serum
FN	- fibronectin
FSH	- follicle stimulating hormone
$\gamma^{32}\text{ATP}$	-radioactive phosphate labelled adenosine triphosphate
HCG	- human chorio gonadotrophin
HITS	- hydrocortisone, insulin, transferrin, sodium selenite
ICRF	- Imperial Cancer Research Fund
IFN	- interferon
IGF-1	- insulin-like growth factor 1
IGF-2	- insulin-like growth factor 2
IU	- international unit(s)
MOPS	- 3-(N-morpholino)propanesulfonic acid
mRNA	- messenger ribonucleic acid
NaPP_i	- sodium pyrophosphate
OD	- optical density
OPD	- orthophenylene diamine
PBS	- phosphate buffered saline

Pg	- progesterone
PVP	- polyvinyl pyrrolidine
RT-PCR	- reverse transcription-polymerase chain reaction
SDS	- sodium dodecyl sulphate
SSC	- saline sodium citrate
TBS	- tris buffered saline
TGF- β	- transforming growth factor-beta
TGF- α	- transforming growth factor-alpha
TN	- tenascin
WHO	- World Health Organisation

Chapter 1

Introduction

1.1 Ovarian Cancer**1.1.1 Epidemiology**

Ovarian cancer, when diagnosed at its earliest stages, is a disease amenable to treatment, with a five year survival rate of approximately 80% (Young *et al.*, 1993). However, as early stage ovarian cancer is asymptomatic the disease is rarely detected until it has spread to other organs (Ozols *et al.*, 1992). Late stage ovarian cancer has a much poorer prognosis with 5 year survival rates of less than 15%. This accounts for the disease statistics that mean of the 5000 cases of ovarian cancer which occur each year in the UK there are 4000 deaths, making ovarian cancer the most common cause of gynaecological cancer death in women.

1.1.2 Etiology of ovarian cancer

Only 5% of all patients with ovarian cancer come from a family with a history of the disease (Greene *et al.*, 1984). This suggests that factors in addition to genetic modifications may induce cancer development in the ovary.

(i) Environmental factors

Many environmental factors potentially contribute to an increased risk in ovarian cancer, but no single factor has been identified as causative. However population studies have strongly suggested that environmental factors may play a role in the process of ovarian carcinogenesis. For example the incidence of ovarian cancer in Japan is low, compared with other industrialised countries. Japanese women who have emigrated to the USA have an increased incidence compared with Japanese in Japan (Lingeman, 1974). It has been proposed that this greater incidence may be due to environmental carcinogens, for example those in the diet.

Higher risk of ovarian cancer has been reported to be associated with increased intake of saturated fats (Cramer *et al*, 1984; Risch *et al*, 1994). Risch *et al*. (1994) also showed that vegetable fibre reduced ovarian cancer risk. However the involvement of single dietary components is always difficult to prove and there have been few confirmatory studies.

Other dietary factors have also been examined; increased alcohol consumption showed no higher risk, while coffee consumption may confer a small risk increase (Byers *et al.*, 1983). The observation that increased lactose consumption from milk products may lead to increased risk (Cramer *et al*, 1989) is still being investigated.

Talcum Powder, which until recently contained asbestos, had been suggested to be involved in ovarian carcinogenesis (Longo and Young, 1979), although, more recent studies have failed to confirm this (Whittemore *et al.*, 1988).

While the link between herpes papilloma virus and cervical cancer has been well established (Vousden, 1989) there is no evidence for a link between viral infections and ovarian cancer (Lingeman, 1974).

(ii) Genetic factors

Three heritable forms of ovarian cancer have been defined. Patients with these hereditary syndromes tend to develop ovarian cancer at a younger age than the general population. Site-specific ovarian cancer and hereditary breast/ovarian cancer are rare and appear to be linked mainly to mutations in the putative tumour suppressor gene BRCA1 (Steichen-Gersdorf *et al*, 1994). Lynch syndrome II associates ovarian cancer with a number of other cancers including colorectal, gastrointestinal and breast carcinoma. Other heritable cases of ovarian cancer are likely to be due to mutations in other genes and a second gene linked to breast and ovarian cancer, BRCA2, has already been identified (Wooster *et al*, 1994).

While 95% of ovarian tumours are not genetically linked through germ cells, they are caused by multiple genetic lesions which accumulate in ovarian surface epithelial cells. Gross chromosomal abnormalities are

evident in ovarian cancer (Gabra and Smyth, 1996), but whether these abnormalities are causal is difficult to determine. Damage to the genome which arises sporadically can be negated by DNA repair mechanisms (some of which are initiated by tumour suppressor genes e.g. p53); however if these tumour suppressor genes themselves become damaged they can no longer programme the repair of any further genetic lesions which may occur. This further damage may result in the activation of oncogenes promoting the process of carcinogenesis.

(iii) Hormonal factors.

Fathalla (1971) proposed that whenever the ovaries release an ovum the damage and proliferative repair processes involved makes the epithelium susceptible to genetic alteration. Cycles of damage and repair could lead to cumulative lesions resulting in an increased risk of development of ovarian cancer. This idea has been referred to as the incessant ovulation theory.

Circumstantial evidence in support of the incessant ovulation theory comes from observations that in nulliparous women there is increased incidence of the disease (Cramer *et al*, 1983). Increased incidence has also been associated with women who have an early menarche or late menopause and are therefore exposed to more ovulatory cycles. The general increase in ovarian cancer could also be partly explained by the decreasing parity of women in developed countries; conversely childbirth seems to confer a protective effect. According to the incessant ovulation theory this protective effect is a consequence of pregnancy preventing further ovulation and thus reducing the risk of genetic damage in the ovary. Other factors which suppress ovulation should therefore also confer a protective effect against ovarian cancer. In this respect studies have now shown that the oral contraceptive pill, which prevents ovulation, can reduce the risk of ovarian cancer by 50%, when taken over a 5 year period (Hankinson *et al.*, 1992).

Other theories of hormonal involvement in the development of ovarian cancer include the excess gonadotrophin hypothesis. Post-menopausal women have increased levels of gonadotrophins and this correlates with the high incidence of ovarian cancer seen in women at the age of menopause (Stadel, 1975). Additionally, some studies have shown that women treated with fertility drugs (which contain gonadotrophins and gonadotrophin stimulating hormones) may have an increased risk of developing ovarian cancer (Whittemore, 1993). As these drugs hyperstimulate the ovary this would increase the number of ovulations and be consistent with the incessant ovulation hypothesis.

(iv) Growth factors and cytokines

The normal ovary undergoes cycles of proliferation and reorganisation that require tight control, not only by hormones but also in an autocrine and paracrine nature by peptide growth factors and cytokines (Giordano, 1992). The loss of regulation by these factors, for example, by constitutive activation of their receptor or over expression of the factor, may give cells a growth advantage resulting in the progression of initiated tumour cells. The most widely investigated group of growth factors in ovarian cancer is the epidermal growth factor (EGF) family (which includes EGF and TGF- α). These factors bind to the EGF receptor which is a member of the type-1 tyrosine kinase family of receptors. Both TGF- α and EGF have been shown to stimulate growth of ovarian cancer cells *in vitro* (Crew *et al.*, 1992) and the EGF receptor appears to be overexpressed in many ovarian tumours (Berchuck *et al.*, 1991). Other members of the type-1 tyrosine kinase family of receptors (including c-erbB-2) are also implicated in ovarian cancer (Berchuck, 1990).

Insulin-like growth factors I and II (IGF-I and IGF-II) are a pair of growth factors which have growth stimulatory effects on ovarian cancer cells *in vitro* (Bartlett *et al.*, 1995). They also play a role in the normal ovarian cycle of replication and differentiation (Giordano *et al.*, 1992).

The transforming growth factor β (TGF- β) family exist in 3 isoforms β_1 , β_2 and β_3 (Arrick, 1990). These factors interact with serine-threonine kinase-linked receptors on the cell surface (Massague, 1992). The TGF- β 's have been shown to inhibit the growth of many ovarian cancer cell lines (Bartlett *et al.*, 1992; Hurteau *et al.*, 1994). Many ovarian cancer cell lines and ovarian tumours express all 3 isoforms (Bartlett *et al.*, submitted). It is likely that TGF- β exerts paracrine effects on the surrounding stromal cells (Dickson *et al.*, 1987).

1.1.3 Pathophysiology

Tumours of the ovary can present with a wider variety of histopathologies than those in other organs; these types have been classified by the World Health Organisation and are summarised in table 1.1.

Table 1.1 Summary of the WHO classification of malignant ovarian tumours (Serov and Scully, 1973)

I	Common "epithelial" tumours
	A. Serous
	B. Mucinous
	C. Endometrioid
	D. Clear cell
	E. Brenner
	F. Mixed epithelial
	G. Undifferentiated
	H. Unclassified
II	Sex cord stromal tumours
III	Lipoid cell tumours
IV	Germ cell tumours
V	Gonadoblastoma
VI	Soft tissue tumours not specific to the ovary
VII	Unclassified tumours
VIII	Secondary metastatic tumours
IX	Tumour-like conditions

The wide range of types of ovarian tumours reflects the complexity of the development of the ovary. However the majority of ovarian tumours (almost 90%) are epithelial in origin, with the remaining tumour types being rare and generally limited to younger women (Li *et al.*, 1973). Common "epithelial" tumours develop in the surface epithelium which is derived from the mesothelium of the embryonic ovary (Fox, 1985). During embryogenesis this mesothelium forms a structure called the Mullerian duct which later develops into the main genital duct. The common epithelial tumours derived from this structure reflect the various histologies into which the Mullerian epithelium can differentiate. Thus, serous tumours are similar to fallopian tube epithelium, endometrioid tumours resemble endometrium and mucinous tumours resemble endocervical epithelium. Of the epithelial ovarian cancers approximately 40% are serous, 15% are endometrioid and a further 15% are mucinous (Young *et al.*, 1993). Clear cell tumour types, which are also similar to the endometrial epithelium constitute only 6% of the epithelial ovarian tumours. 17% of epithelial ovarian tumours are undifferentiated and these tumours tend to be very aggressive.

Benign tumours are usually cystic and of serous or mucinous histology (Ashley, 1990). They can grow to enormous size but do not invade their underlying stroma. Consequently benign tumours can simply be removed by surgery and have an excellent prognosis.

A small group of tumours is classified as having borderline malignant potential. Generally, these tumours are well differentiated, show no destructive stromal invasion, progress slowly and rarely metastasise. The term "borderline tumours" was chosen by the WHO; however, FIGO prefers the designation "carcinoma of low malignant potential". Like benign tumours, these have an excellent prognosis.

1.1.4 Stage and grade

The stage at which ovarian cancer is detected is very important to the outcome of the disease. The stage is determined by the extent of disease at the time of diagnosis and the most widely used staging classification is that of the International Federation of Gynaecology and Obstetrics (FIGO) which is shown in table 1.2 (FIGO Committee, 1986).

The survival of patients is directly correlated with their tumour stage. The 5 year survival for patients with stage I disease is 80-90%, and patients with stage II disease also have a good 5 year survival rate (approximately 80%). Patients with later stage disease have a poorer prognosis with 5 year survival rates of approximately 15% for stage III and less than 5% for stage IV (Ozols *et al.*, 1992).

In addition to the stage of the cancer being significant, the degree of histological differentiation of the tumour is of prognostic value. The grading system in widespread use was originally proposed by Broders in 1926. Grading is based on the percentage of undifferentiated cells in the tumour section: well differentiated tumours demonstrate very little cellular atypia (0-25%), moderately differentiated tumours still show a small degree of histological differentiation but the proportion of undifferentiated cells is increased. Poorly differentiated tumours may be totally undifferentiated and are frequently rapid growing and very aggressive (Day *et al.*, 1975).

Table 1.2 FIGO stages for primary carcinoma of the ovary

Stage	Definition
I	Growth limited to the ovaries
IA	Growth limited to one ovary; no ascites; no tumour on the external surface; capsule intact
IB	Growth limited to both ovaries; no ascites; no tumour on the external surface; capsule intact
IC	Tumour either stage Ia or Ib but with tumour on surface of one or both ovaries; or with capsule ruptured; or with ascites present containing malignant cells or with positive peritoneal washings
II	Growth involving one or both ovaries with pelvic extension
IIA	Extension and/or metastasis to the uterus and/or tubes
IIB	Extension to other pelvic organs
IIC	Tumour either stage IIa or IIb but with tumour on surface of one or both ovaries; or with capsule ruptured; or with ascites present containing malignant cells or with positive peritoneal washings
III	Tumour involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes; superficial liver metastasis; tumour is limited to the true pelvis but with histologically proven malignant extension to the small bowel or omentum
IIIA	Tumour grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces
IIIB	Tumour involving one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces not exceeding 2 cm in diameter; nodes are negative
IIIC	Abdominal implants greater than 2 cm in diameter and/or positive retroperitoneal or inguinal nodes
IV	Growth involving one or both ovaries with distant metastases. If pleural effusion present, there must be positive cytology to assign a case to stage IV; parenchymal liver metastasis.

1.1.5 Treatment of ovarian cancer

(i) Surgery

Initial surgery is vital for accurate diagnosis and staging of ovarian cancer, as the stage of the disease determines the nature of further treatment. In early stage disease surgery alone may be adequate treatment with no further therapy being required (Young *et al.* 1990). Although unlikely to be curative, primary surgery is often performed in patients with more advanced stage cancer, as debulking of residual tumour mass not only increases the patients comfort but may also result in increased survival. Debulking to less than 2cm improves the chance that the tumour will respond to adjuvant therapy (Delgado *et al.* 1984).

(ii) Radiotherapy

Radiotherapy is not frequently used for the treatment of ovarian cancer, as the entire abdomen requires irradiation and this can result in serious complications, due to the sensitivity of the liver, kidneys and bowel to radiation. However this approach may be used in patients with early stage disease with poor prognostic factors, who have received good debulking surgery (Ozols *et al.*, 1992).

(iii) Chemotherapy

Ovarian cancer was one of the first solid tumours to be treated with systemic cytotoxic chemotherapy (Ozols and Young, 1984). Systemic therapy is now widely accepted as the best treatment for patients whose ovarian cancer extends beyond the pelvis (stage III and IV). Twenty years ago the treatment of choice was single alkylating agent therapy, usually melphalan. Several non-alkylating agents have been found to be active against ovarian cancer, with cis-diamminedichloroplatinum (cisplatin) probably being the most active agent for the treatment of ovarian cancer (Thigpen, 1985). However, cisplatin causes a variety of adverse side effects. Problems with toxicity of cisplatin led to the development of analogues such as carboplatin.

Standard chemotherapy now consists of platinum containing regimens in combination with other drugs (usually an alkylating agent). These produce a higher response rate than single agent regimens (Young *et al.* 1978) but the overall 5 year survival rate of patients remains low. It is generally believed that this is due to acquired or inherent drug resistance, and thus new therapies are constantly being sought. One new drug which has been shown to improve outcome, when used in combination with platinum, is Taxol. Taxol produces mitotic arrest and is currently being examined in prospective clinical studies (Einzig *et al.*, 1992; Kavanagh *et al.*, 1993).

(iv) Immunotherapy

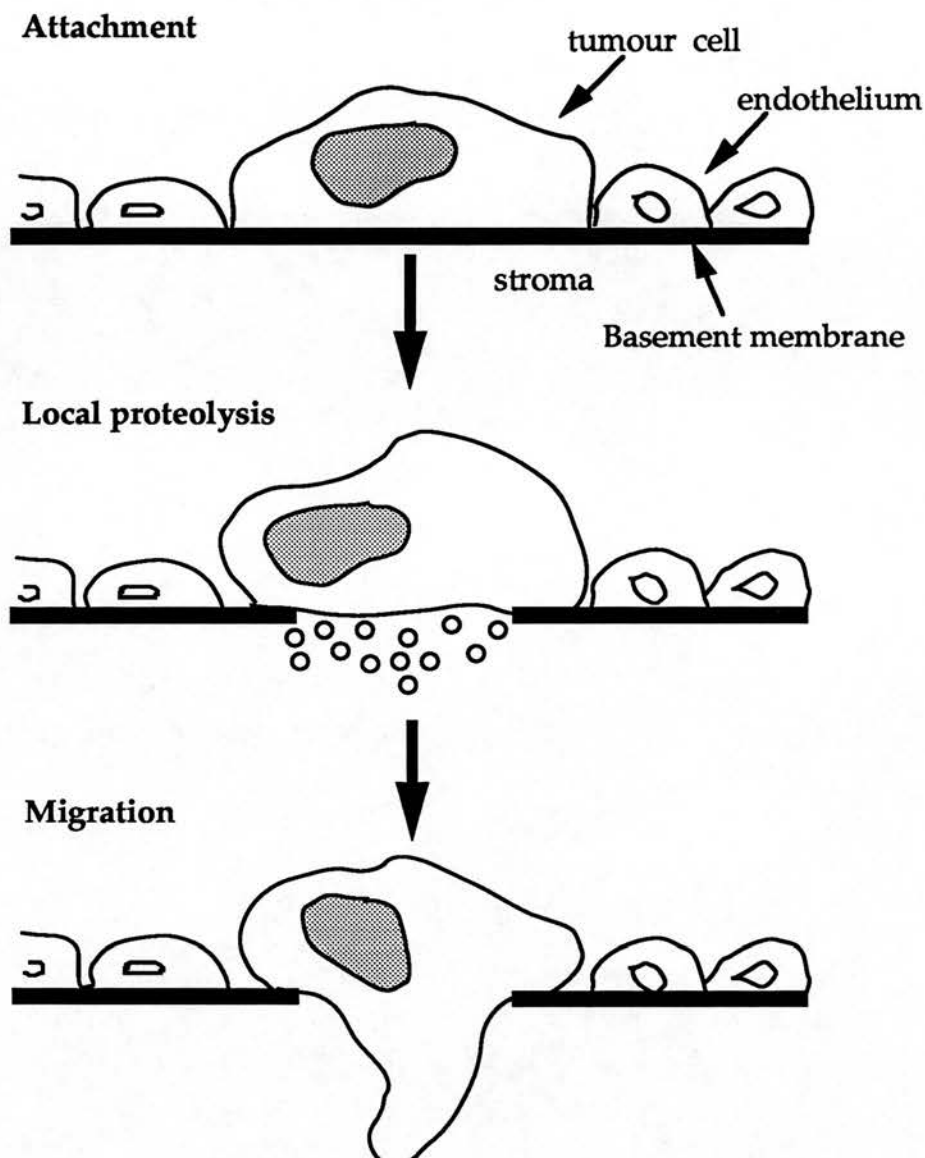
The containment of ovarian cancer within the peritoneal cavity makes it a suitable target for immunotherapy. To date most studies have utilised non-specific immunotherapy, often employing cytokines to stimulate the host's immune response. A few studies have utilised specific monoclonal antibodies, coupled to cytotoxic moieties, however these can produce serious side effects. These strategies and other current experimental therapies, such as genetic manipulation of tumour cells to make them more immunogenic, are reviewed by Kehoe (1995).

1.2 Invasion and metastasis

The ability of malignant tumours to spread from their original site and form secondary tumours in distant tissues presents the greatest obstacle to successful tumour treatment. The process by which tumour cells invade and metastasise is a complex multistage set of interactions involving tumour cells, host cells and extracellular matrix (Aznavorian *et al.*, 1992; Liotta *et al.*, 1991). The metastatic process (figure 1.1) begins with the tumour cells penetrating the epithelial basement membrane and invading the surrounding stroma. To travel to distant sites the tumour cells have to then enter either the blood or lymphatic system. Once in these systems the tumour cells must survive the body's immune response, arrest in a vascular bed, invade and proliferate in a secondary tissue to form a new tumour.

The first stages of the metastatic process, involving interactions with the matrix, can be separated into 3 defined sequential steps, namely attachment, proteolysis and migration. These stages are discussed separately in the sections below.

Figure 1.1 Three-step hypothesis of invasion



This figure (adapted from Aznavoorian *et al.*, 1992) illustrates the three-step hypothesis of invasion. The first step shows a tumour cell in a capillary attached to the subendothelial membrane. The second stage involves the cleavage of matrix components by the secretion/activation of proteinases. Finally the tumour cell migrates into the stroma, beginning with the protrusion of pseudopodia.

1.2.1 Tumour cell adhesion

In order to progress the invasive process, tumour cells must first do two things; (i) detach from neighbouring cells and (ii) form an attachment with the basement membrane surrounding the tumour. Cell adhesion molecules such as cadherins, which mediate cell-cell binding, have been shown to have inhibitory roles in the process of metastasis (Uleminckx *et al.*, 1991). Some studies looking at adhesion of tumour cells to matrix proteins, through receptors such as integrins have shown that cells with strong adhesion to the substrata are less invasive (Giancotti and Ruoslahti, 1990). Other investigations have shown that inhibition of tumour adhesion to matrix proteins also results in less aggressive invasive behaviour (Aznavorian *et al.*, 1992). While these results appear to be contradictory, Aznavoorian *et al.* (1992) postulated that invasion may actually require an intermediate level of adhesion to the matrix. Cells which adhere strongly may never break away from their original site, while those which are unable to attach to the matrix may not digest and migrate through the basement membrane, and would not arrest in and invade a second site.

1.2.2 Proteolysis

The basement membrane is a continuous, dense matrix of protein which must be breached if tumour cells are to invade. Two to eight hours after tumour cells have attached to the basement membrane, a localised zone of lysis is seen in the matrix at the point of tumour cell contact. The main family of proteolytic enzymes implicated in the process of matrix dissolution are the matrix metalloproteinases. These enzymes are secreted in the form of proenzymes which require activation. The family of matrix metalloproteinases can be divided into 3 groups (Matrisian 1992): the stromelysins, interstitial collagenase and type IV collagenases. The groups are categorised on the basis of substrate specificity. Interstitial collagenase degrades fibrillar collagens (Templeton *et al.*, 1990) and the

type IV collagenases are capable of degrading type IV collagen which is the major component of basement membrane (Collier *et al.*, 1988; Hibbs *et al.*, 1987). The stromelysins, stromelysin-1, stromelysin-2, and matrilysin can degrade laminin, fibronectin and proteoglycan core proteins (Wilhelm *et al.*, 1987). Another member of this family is named stromelysin-3 on the basis of its homology with the other enzymes but its substrate specificity is yet to be elucidated (Basset *et al.*, 1990). The other three known classes of proteinases (serine, aspartyl and cysteinyl) have also been implicated in the process of matrix proteolysis. Of most interest is the serine protease, urokinase-type plasminogen activator. The overexpression of this enzyme has been related to enhanced invasion and metastasis (Dano *et al.*, 1985).

The expression of these proteases is not limited to invasive tumours as proteolysis also occurs in normal tissues, during tissue remodelling and in other situations such as bacterial infections. The expression of the enzymes is highly regulated and even during the process of tumour invasion the degradation of the matrix occurs in a controlled fashion as matrix components are required for the next stage of the process, namely cell migration. The difference between normal and tumour cells is that, in malignancy the process of membrane degradation is occurring at a time and place which would be inappropriate for normal cells. Tumour cells are also able to produce some of these enzymes themselves (Gottesman, 1990), or induce other cells to secrete them. The enzymes are secreted in a highly localised manner until the active enzyme outbalances the natural tissue inhibitors of metalloproteinases (TIMPS) (Carmichael *et al.*, 1986).

1.2.3 Cell migration

The third step in the invasion of the interstitial stroma involves the locomotion of the tumour cells through the basement membrane. The importance of tumour cell motility in invasion and metastasis was first demonstrated by Corman in 1953. Cell migration again is a highly coordinated process which can occur in certain normal situations (Lauffenburger and Horwitz, 1996). The process begins with the morphological polarisation of the cell followed by membrane extension at the leading edge to form pseudopodia. The extended part of the cell must then form attachments to the matrix and produce a contractile force to move the cell forward. This contractile force appears to be generated by active myosin based motors. For forward movement the attachment of the rear of the cell to the matrix must be released and the receptors binding the matrix are either lost or recycled.

Migration of tumour cells can be promoted by a number of factors including matrix components (McCarthy and Furcht, 1984), growth factors (Stracke *et al.*, 1988) and scatter factors (Weidner *et al.*, 1990). These can stimulate motility either randomly (chemokinesis); in a directional fashion whereby cells move in response to a gradient of factor concentration (chemotaxis); or in a directional manner towards substrate bound ECM proteins (haptotaxis). Tumour cells themselves can also produce autocrine motility factors (Liotta *et al.*, 1986).

1.2.4 Integrins

The processes of cell attachment and migration involve receptors on the cell surface. The integrins are a large family of cell surface adhesion receptors. The original classification of integrins recognised 11 different human integrins (Hynes, 1987) but there are now 21 different identified integrins (Yokosaki and Sheppard, 1995).

Structurally all integrins consist of two non-covalently linked subunits. The α subunits range from 120 to 180 kDa and are linked to a β subunit of

molecular weight 90 to 110 kDa. Both subunits are type I transmembrane glycoproteins, composed of a large extracellular domain, a hydrophobic membrane spanning region and a short cytoplasmic domain. The heterodimers are formed by association of the extracellular domains. The pivotal roles of integrins are to mediate cell-cell and cell-matrix interactions and both subunits contain ligand binding sites in their extracellular domains. The α subunit also contains a binding site for metal cations. Integrins recognise active site peptides on the ECM proteins, the best characterised of which is the tripeptide Arg-Gly-Asp (RGD). The RGD site in fibronectin and tenascin has been analysed and shown to exist in a flexible loop structure (Main *et al.*, 1992; Leahy *et al.*, 1992). The two ligand binding sites on the integrin subunits are closely associated, therefore the substrate specificity of the integrin is determined by both the α and β subunits. Integrins can be monospecific (e.g. $\alpha 6\beta 1$, which recognises only laminin), or polyspecific (e.g. $\alpha 3\beta 1$, which can bind fibronectin, collagen laminin and epilgrin); this results in considerable redundancy in ligand recognition (Hynes, 1992). Table 1.3 shows the specificity of integrins for selected extracellular matrix proteins.

The specificity of integrins is generally established using purified subunits, but it can vary depending upon the cell type on which they are expressed (Kirchofer *et al.*, 1990). It has also been observed that the specificity and affinity of integrins on a given cell type is not constant. Masumoto and Hemler (1993) demonstrated that cells can activate or deactivate their integrins. Pullman and Bodmer (1992) identified a cytoplasmic protein which increased the levels of $\alpha 2\beta 1$ -mediated adhesion to collagen. Integrin function can therefore be regulated from inside the cell (inside to out signalling) or by binding of external factors such as matrix proteins (outside to in signalling). There is increasing evidence that integrins not only act as molecular anchors, but they are also capable of transducing signals from a cell's environment via the cytoplasmic domains of the α subunits, which are linked to the

cytoskeleton. A direct link has been demonstrated between integrins and tyrosine phosphorylation of the phosphoprotein focal adhesion kinase (Humphries *et al.*, 1993).

Table 1.3 Specificity of integrins for selected ECM proteins
(Adapted from Yokasaki and Sheppard, 1995)

Fibronectin	Collagen	Tenascin
	$\alpha 1\beta 1$	
	$\alpha 2\beta 1$	$\alpha 2\beta 1$
$\alpha 3\beta 1$	$\alpha 3\beta 1$	
$\alpha 4\beta 1$		
$\alpha 5\beta 1$		
$\alpha 8\beta 1$		$\alpha 8\beta 1$
		$\alpha 9\beta 1$
$\alpha \text{IIb}\beta 3$		
$\alpha \text{V}\beta 1$		
$\alpha \text{V}\beta 3$	$\alpha \text{V}\beta 3$	$\alpha \text{V}\beta 3$
$\alpha \text{V}\beta 6$		$\alpha \text{V}\beta 6$
$\alpha 4\beta 7$		

1.3 Tenascin

It has been known for many years that extracellular matrix (ECM) molecules play vital roles in organ morphogenesis (Grobstein, 1954). Many ECM components have been identified, including proteoglycans, collagens and other glycoproteins. Study of these proteins has shown that they are not merely structural proteins but they can also bind to cellular receptors, such as integrins, and affect cell function (McDonald, 1989). Many ECM molecules are ubiquitous, but the large ECM glycoprotein tenascin (TN) has a limited distribution and is often only seen in the mesenchymal ECM at times of important tissue interactions; during embryogenesis, when tissues are reorganising or regenerating (as in healing wounds) and also in tumours.

1.3.1 Discovery and nomenclature of tenascin

In hindsight it can be seen that the first reference to TN was actually made by Yamada *et al.* in 1975; this group purified cell surface fibronectin and assigned it the property of haemagglutination. It was noted in 1978 by Alexander *et al.* that the fibronectin preparation contained disulphide-bound oligomers, and in 1984 Erickson and Inglesias used rotary shadowing electron microscopy to examine these oligomers and found that they had a distinctive structure of 6 long flexible arms and that the structure of these arms was different to that of fibronectin. Erickson and Inglesias concluded this molecule was a new protein and called it hexabrachion.

At the same time as Erickson and Inglesias were carrying out work on the fibronectin preparations, Chiquet and Fambrough described a new protein which they had discovered in connective tissue. This protein too was a hexameric glycoprotein and they termed it myotendinous antigen (Chiquet and Fambrough, 1984). Another group was independently looking for proteins associated with tumours and produced an antibody to an ECM protein which was prominent in the stroma and vasculature

and appeared to be absent in normal tissues. They named their protein GMEM (glioma mesenchymal extracellular matrix) and demonstrated that it was a disulphide bound oligomer (Bourdon *et al.*, 1985). Two neurobiology groups also described similar proteins which they named J1 (Kruse *et al.*, 1985) and cytotactin (Grumet *et al.*, 1985).

It eventually became apparent that these diverse studies had identified the same protein as the homology became obvious. The different areas of discovery painted a picture of a protein with a very interesting distribution.

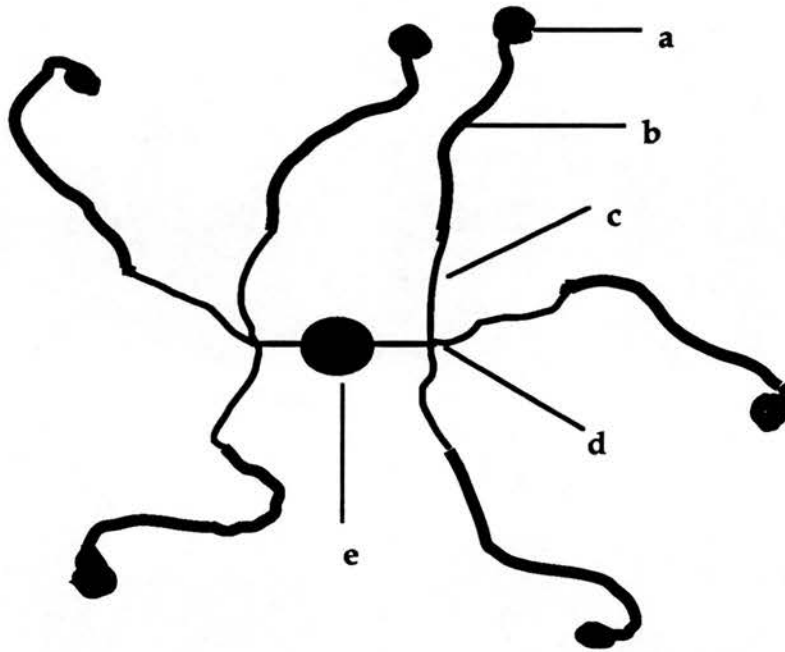
In 1986 Chiquet-Ehrismann *et al.* demonstrated a link between the novel ECM protein and tumours. In their paper they also proposed the name tenascin for this protein. The name is derived from the combination of the latin verb "tenere" (to hold) and "nasci" (to be born) reflecting partly its discovery by Chiquet and Fambrough in tendons and also its embryogenic expression. The name tenascin is now universally accepted.

1.3.2 Structure of tenascin

(i) Hexamer structure

Probably the most distinctive structural feature of TN is its hexabrachion structure, which can be clearly seen in the rotary shadowed electron micrographs (Erickson and Lightner, 1988). An illustration of this hexameric structure can be seen in figure 1.2.

Figure 1.2
The structure of the TN hexamer



The hexabrachion can be seen to have five distinct features: (a) a terminal knob, (b) a thick distal segment, (c) a thinner inner segment (d) a "T"-junction where three arms are connected by formation of a triple coiled coil of the 3 arms and finally (e) a central globular particle which connects the 2 trimers by a disulphide bridge to form the hexabrachion structure. The hexamers are in fact assembled by a novel co-translational process which is facilitated by the length of the subunits (Redick *et al.*, 1995).

This hexabrachion is a very large protein whose size is around 2000 kDa. The protein can be purified from conditioned media using immunoaffinity chromatography (Chiquet-Ehrismann *et al.*, 1986) or by standard biochemical protein purification methods the most important stage of which involves separation of TN according to its large size.

(ii) Monomer structure

The TN molecule is a homohexamer and each monomer is built up of characteristic structural domains, as shown below in figure 1.3.

Figure 1.3

The structure of the TN monomer



The TN monomer consists of 5 distinct domains some of which show homologies to other proteins (Jones *et al.*, 1989). Firstly, an N terminal domain (), which can crosslink the monomers by disulphide bonds. This domain is followed by heptad repeats which participate in the formation of the trimer structure. The flexible arms of the hexabrachion molecule consist of $14\frac{1}{2}$ epidermal growth factor (EGF) like repeats (♦), up to 16 fibronectin type III repeats () and finally a COOH-terminal globe which is homologous to the globular domain of the β and γ chains of human fibrinogen.

TN is encoded by a single gene, which has been cloned and sequenced (Gulcher *et al.*, 1991; Nies *et al.*, 1991) and its expression is regulated by a single promoter which has been shown to contain a number of response elements (Jones *et al.*, 1990) including sites for homeobox genes and their encoded proteins (Jones *et al.*, 1992). It has also been shown that the first untranslated exon of the gene plays a role in the regulation of TN gene expression (Gherzi *et al.*, 1995).

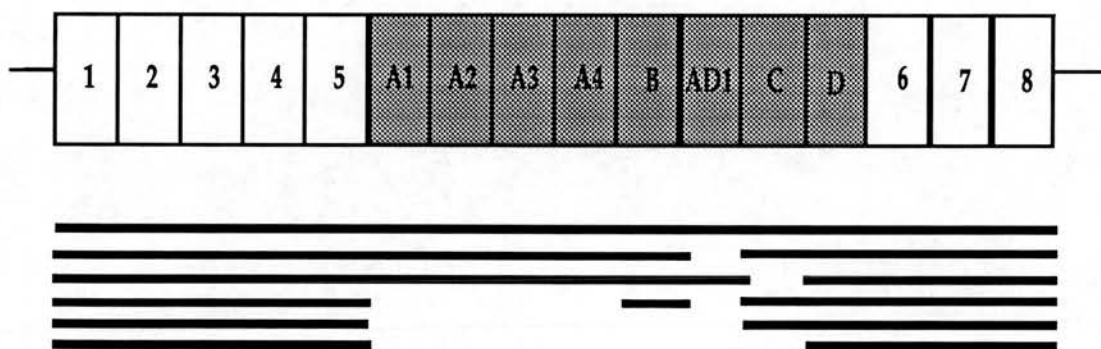
(iii) Alternative splicing

Many eukaryotic genes contain extra sequences which are not present in their corresponding mRNA; these non-coding sequences are called introns and the coding sequences are called exons. The primary RNA transcript of these genes contains the intron sequences which are then spliced out in a precise multistep process to produce the mRNA which leaves the nucleus. If every exon in the gene is present in the processed mRNA then this is known as constitutive splicing and only one protein is produced from the gene. If individual exons are excluded from some transcripts and included in others this is known as alternative splicing.

Alternative splicing provides a means of forming a set of proteins that are variations on a basic motif.

The TN primary mRNA transcript undergoes alternative splicing in its region of FN type III repeats as illustrated in figure 1.4.

Figure. 1.4 Alternative splice forms of the FN type III repeats of TN.



This figure (adapted from Chiquet-Ehrismann, 1993) illustrates the FN type III repeats of the TN monomer. The shaded boxes represent the repeats which can undergo alternative splicing. The lines beneath the diagram indicate possible splice variants, which have been previously reported in the literature.

Gulcher *et al.* (1989) first demonstrated that the human TN gene underwent splicing and indicated that some forms contained 7 extra FN type III repeats. Siri *et al.* (1991) detected 4 of the above variants using reverse transcription-polymerase chain reactions. That the additional AD1 repeat existed in some TN variants was discovered by Sriramarao and Bourdon in 1993.

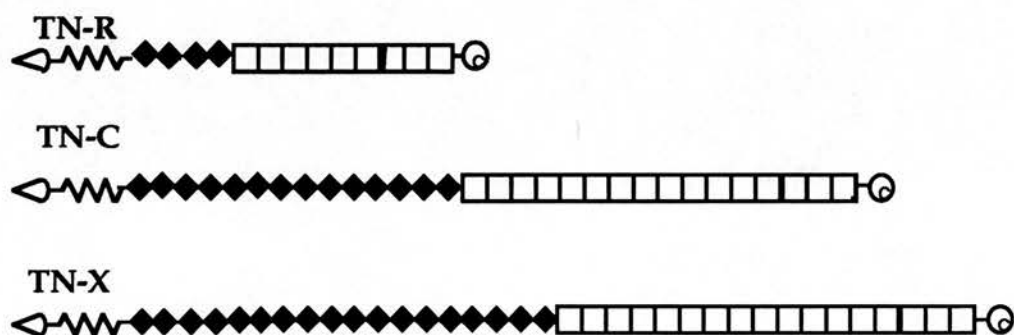
Which of these RNA splice variants go on to be translated into proteins is unclear. To date, most groups have reported 2 predominant forms of TN; large (approximately 300 kDa) and small (approximately 200 kDa). However there is some evidence that tumour cells may be able to produce intermediate size forms of the protein. Larger isoforms of TN are more frequently associated with proliferating tissues.

(iv) The TN family

Two new proteins have been discovered that are related to TN, consisting of similar domains and potentially having similar functions. A new nomenclature has been proposed by Erickson (1993) to distinguish between the members of this family of TNs. The original TN has been designated TN-C, the C stands for cytotactin, which was the name associated with the first published sequence. The two new family members are named TN-R and TN-X. TN-R was discovered as restrictin in the chicken nervous system (Rathjen *et al.*, 1991) and in the rat the same protein was identified and named J1-160/180 (Pesheva *et al.*, 1989). Both proteins have subsequently been cloned (Norenberg *et al.*, 1992; Fuss *et al.*, 1993). The TN-X gene was discovered in the human major histocompatibility complex class III locus and has since been sequenced by Bristow *et al.* (1993).

The basic structure of these 3 main family members is shown in figure 1.5

Figure 1.5 The TN family



Each form of TN contains the C-terminal fibrinogen like domain and various numbers of FN type III repeats ranging from 9 possible repeats in TN-R to 29 repeats in TN-X. The final FN type III domain is conserved in all 3 proteins.

It is very likely that more members of the family will be identified. Chiquet-Ehrismann *et al.* (1994) have discovered in chicken the partial DNA sequence of a new member which they have tentatively termed TN-Y and believe they have further evidence for the existence of other TN-like genes. Research has also identified 2 proteins in *Drosophila*, which may be invertebrate TN.

1.3.3 Distribution

The main interest in TN sprang from observation of its spatial and temporally restricted distribution pattern. During normal embryogenesis TN is prominent in the developing nervous system, in matrix lining the pathway of migrating cells, in mesenchyme at sites of epithelial-mesenchymal interaction and in developing connective tissue. The original discoveries of TN in developing organs and in tumours lead to

the belief that TN may be a true oncofoetal protein. This belief appeared to have been substantiated by Mackie *et al.*, (1987), who showed that TN was only present in the stroma of malignant breast tissue and was not seen in benign or normal samples. However it has since become apparent that the antibody used to detect TN recognised part of the alternatively spliced FN type III repeats and was therefore detecting only the largest isoforms of the molecule (Borsi *et al.*, 1992).

Since this discovery TN has been detected in some normal tissues but at much lower levels than in foetal counterparts. In cases of disease or injury, expression can, however, be greatly increased (see references below).

(i) Normal tissues

TN is most strongly expressed in regenerative organs such as the gut (Aufderheide and Ekblom, 1988) and the skin (Lightner *et al.*, 1989) in which the epithelium is being constantly renewed. Lower levels of expression have been detected in other adult organs and tissues. Borsi *et al.* (1993) detected TN mRNA in a wide range of tissues and organs including heart, brain, lung, liver and kidney. Ventimiglia *et al.* (1992) also demonstrated TN protein in normal adult brain and kidney. TN is also found in the smooth muscle wall of blood vessels (Mackie *et al.*, 1992).

Interestingly, a marked increase in TN expression can be seen when cellular proliferation and reorganisation are occurring. The most obvious example of this phenomenon is in healing wounds in which the levels of TN are markedly increased at the dermal-epidermal junction beneath the regenerating epidermis (Mackie *et al.*, 1988). TN expression is also increased in hyperproliferative skin diseases such as psoriasis (Schalkwijk *et al.*, 1991).

In the normal adult breast, which was previously thought not to express TN, it has been shown that TN can be detected during gestation and

lactation (Howeedy *et al.*, 1990). Levels of TN expression in normal breast vary according to the stage of the menstrual cycle, with the highest level of TN expression at day 22 of the cycle which corresponds to the peak in mitosis (Ferguson *et al.*, 1990).

Tamura *et al.* (1993) have demonstrated the presence of TN in normal ovary by immunohistochemistry. The expression of the protein varied widely according to the stage of the menstrual cycle. Little staining was observed in preovulatory follicles, and only low levels of expression were seen in functioning corpora lutea. Ovaries undergoing reorganisation, caused by the involution of the corpora lutea, were associated with much stronger TN staining.

(ii) Tumours

Since the original findings of increased expression of TN in gliomas (Bourdon *et al.*, 1985) and breast tumours (Mackie *et al.*, 1987), overexpression of TN has been reported in many other solid tumours by immunohistochemistry. A summary of the tumours studied is shown in table 1.4.

All the cases investigated showed that there was an increased incidence of intense and diffuse staining, in the ECM of the stromal mesenchyme, in malignant tumours, when compared to normal or benign tissue. For example Ibrahim *et al.* (1993) demonstrated, in human prostate tissue, that while most samples studied were positive for TN staining (80% of benign and 96% of malignant), the benign tissue generally did not show the strong stromal staining and instead expressed TN in a focal periglandular fashion.

Table 1.4

Tumours shown to overexpress TN

Tumour type	Reference	
glioma	Bourdon <i>et al.</i>	1983
basal cell carcinoma	Stamp <i>et al.</i>	1989
endometrium	Vollmer <i>et al.</i>	1990
melanoma	Herlyn <i>et al.</i>	1991
breast	Koukoulis <i>et al.</i>	1991
lung	Oyama <i>et al.</i>	1991
colon	Reidl <i>et al.</i>	1992
liver	Yamada <i>et al.</i>	1992
prostate	Ibrahim <i>et al.</i>	1993
salivary gland	Shrestha <i>et al.</i>	1994
astrocytoma	Zagzag <i>et al.</i>	1995

However the expression of TN has not been examined in ovarian tumours.

1.3.4 The nature of the cells producing tenascin.

It has generally been assumed that the cellular source of TN in tumours is the stromal cells surrounding the epithelium. This is mainly because of observations of immunohistochemical staining patterns and experiments with epithelial cells in culture (Sakai *et al.*, 1993). It has since been shown that epithelial cells are capable of producing TN *in vivo*. Lightner *et al.* (1994) demonstrated by in situ hybridisation that epithelial cells are an important source of TN in both normal and malignant breast tissue. Other studies have shown that many epithelial cells are capable of secreting TN *in vitro* (Kawakatsu *et al.*, 1992).

Whatever the cellular source of TN it is clear that increased levels are seen where there are epithelial-mesenchymal interactions. Several findings have suggested that TN synthesis is induced in the stroma as a result of interactions with neoplastic epithelium. Chiquet-Ehrismann *et al.* (1989) demonstrated that conditioned media from the MCF-7 breast carcinoma cell line induced TN synthesis in fibroblasts. More recently it has been shown that the converse can also occur and fibroblasts can induce TN synthesis in epithelial cells (Hiraiwa *et al.*, 1993; Sakai *et al.*, 1995).

1.3.5 Regulation of tenascin synthesis

(i) Growth factors and cytokines

It is clear, from TN's temporally and spatially restricted distribution pattern, that the expression of this protein is tightly regulated. Epithelial-mesenchymal interactions have been shown to induce TN synthesis, and this induction does not rely solely on direct contact between the cells but may be mediated by soluble factors (Hiraiwa *et al.*, 1993).

Serum is a potent inducer of TN and fractionation by heparin affinity chromatography has provided several active fractions, indicating that there are several factors capable of inducing TN in serum (Vrucinic-Filipi and Chiquet-Ehrismann, 1993).

Many growth factors and cytokines have been tested and shown to induce TN synthesis. These include: TGF- β_1 (Pearson *et al.*, 1988), EGF (Sakai *et al.*, 1995), FGF (Tucker *et al.*, 1994), IL-1 and IL-4 and TNF- α (Rettig *et al.*, 1994), activin (Umbhauer *et al.*, 1992), PDGF (Sharifi *et al.*, 1992) and angiotensin II (Mackie *et al.*, 1992). The effects of many of these factors are cell specific, showing the dependence of a response on the presence of receptors and requirement for second messenger pathways.

(ii) Hormones

There is some evidence that TN synthesis may be regulated by hormones. Vollmer *et al.* (1994) showed that hormones such as progesterone could affect TN synthesis indirectly, by altering the proliferative activity and degree of differentiation of the epithelium. They found that a low degree of differentiation induced TN expression.

Glucocorticoid hormones have been shown to inhibit TN expression in bone marrow and fibroblast cells (Ekblom *et al.*, 1993).

(iii) Mechanical stress

Chiquet-Ehrismann *et al.* (1994) showed that mechanical stress, generated by culturing cells in restrained collagen gels, could induce TN secretion in fibroblasts.

1.3.6 The search for a function

The association of TN expression with epithelial proliferation and morphogenesis, as well as its overexpression in tumours and healing wounds has lead to speculation that TN plays a significant role in regulating cell function. *In vitro.* studies have shown TN is capable of producing varying effects on the behaviour of cell lines.

(i) Adhesion and spreading

Because of TN's modular structure and homology to fibronectin, many studies of the protein have investigated its role in cell adhesion. One of the most widely reported properties of TN is its "anti-adhesive" activity. Chiquet-Ehrismann *et al.* (1989) and Kawakatsu *et al.* (1992) found that MCF-7 breast carcinoma cells adhere poorly to TN and Herlyn *et al.* (1991) showed that TN is also a poor substrate for melanoma cell adhesion.

Chiquet-Ehrismann *et al.* (1988) showed that TN could interfere with the adhesive action of FN, inhibiting integrin mediated attachment of cells to this protein. Both Chiquet-Ehrismann *et al.* (1991) and Chung *et al.* (1995) have demonstrated that TN can physically bind to FN. Lightner *et al.*

(1990) showed TN can possibly interfere with cell binding to ECM proteins sterically blocking cell access to the matrix.

However, TN has also been shown to have adhesive properties. Spring *et al.* (1989) used recombinant fragments to elucidate how TN could be both adhesive and anti-adhesive. They observed that there was a strong cell binding site at the tip of the TN arm while, in contrast, the fragment containing the EGF-like repeats showed an anti-adhesive effect.

Many studies have also demonstrated that TN can promote cell attachment but inhibit cell spreading (Chiquet-Ehrismann *et al.*, 1988; Spring *et al.*, 1989); this may indicate that the signals for these two processes exist on different parts of the molecule and may be mediated by different receptors.

The contrasting reports in the literature indicate that TN's effects on adhesion and spreading are cell type specific, but it should also be noted that the source of TN used in these different studies has not been uniform and it is very likely that different isoforms of TN will have different properties. In support of this Murphy-Ullrich *et al.* in 1991 showed that only the larger form of TN-C resulted in loss of focal adhesion of epithelial cells.

(ii) Migration

In developing embryos TN is located along the pathway of migrating cells particularly in the developing nervous system (Mackie *et al.*, 1988). Again experiments examining the effects of TN on migration *in vitro* have produced apparently contradictory results. Giese *et al.* (1995) showed that TN is a permissive substance for glioma cell migration; Deryugina and Bourdon (1996) demonstrated the same effect and also demonstrated that TN modulated cell migration on FN. Hoffman *et al.* (1994) found that TN inhibited the migration of monocytes through a matrigel coated porous membrane. Thus it appears that the effects of TN on cell migration may be cell specific.

(iii) Other effects of TN

TN expression is enhanced in regions of increased proliferation, and the TN molecule contains regions homologous to the growth factor EGF; these observations suggest that TN may be capable of acting as a growth factor. TN has been shown to be growth stimulatory to rat tumour cells (Chiquet-Ehrismann *et al.*, 1986). End *et al.* (1993) demonstrated TN could stimulate the growth of quiescent Swiss 3T3 fibroblasts; they also showed that the TN molecule was internalised in these cells. In contrast Crossin (1991) showed that TN inhibited DNA synthesis in rapidly proliferating 3T3 cells.

Expression of TN may also correlate with angiogenesis (Zagzag *et al.*, 1995). As angiogenesis is a process which involves cell movement and reorganisation, and TN is expressed in the vascular smooth muscle wall, it seems likely that TN's adhesive and migratory properties may be involved in this process. Hahn *et al.* (1995) showed that TN provided a substratum for directed endothelial and smooth muscle cell migration. Additionally Canfield and Schor (1995) described how TN can promote endothelial cell sprouting; this is a transition from a resting phenotype to an angiogenic phenotype.

TN has been shown to be capable of inducing genes such as c-fos and several proteinases including 92 kDa gelatinase, stromelysin and collagenase (Tremble *et al.*, 1994). Interestingly, TN can be digested by many proteinases including stromelysin (Imai *et al.*, 1994). These effects were only seen in the context of a matrix of mixed substrates and were not mediated by soluble ECM proteins. Chiquet-Ehrismann (1991) states that ECM proteins which disrupt cell adhesion or alter cell morphology could greatly effect cellular function by causing the release of cytoskeleton associated factors of second messenger pathways. Different cell types would, therefore, produce different responses to the ECM depending on the receptors, cytoskeleton components and second messenger systems present. TN may also be involved directly in intracellular signalling through its cell surface receptors, described in section (iv) below.

Additional functions of TN have been shown to include alteration of cellular pH (Krushel *et al.*, 1994), immunosuppressive activity (Ruegg *et al.*, 1989) and differentiation of mammary cells (Chammas *et al.*, 1994). Vollmer (1994) speculates that TN may be expressed in reorganising tissues as a "survival factor" suppressing apoptosis.

(iv) TN receptors

TN was shown to mediate cell attachment by interacting, through an arginine-glycine-aspartic acid (RGD) tripeptide, with a cell surface receptor (Bourdon and Ruoslahti, 1989). This indicated that TN interacted with integrin type receptors as described in section 1.2.4. TN has since been shown to interact with several integrins, including $\alpha 2\beta 1$ and $\alpha V\beta 3$ (Sriramarao *et al.*, 1993), $\alpha V\beta 6$ (Prieto *et al.*, 1993), $\alpha 8\beta 1$ (Schnapp *et al.*, 1995) and $\alpha 9\beta 1$ (Yokosaki *et al.*, 1994). These integrins may be present on different cell types and have different functions. The relevance of integrins to TN is reviewed by Yokosaki and Sheppard (1995).

Several other candidates for TN receptors have also been proposed including F11/ contactin, which is a member of the Ig super-family of cell surface/adhesion molecules, and is abundant on neurons (Vaughan *et al.*, 1994). Annexin II was identified as a high affinity receptor for TN by Chung and Erickson in 1994. It appears to be a receptor for the alternatively spliced FN type III domains and may mediate cellular responses to soluble TN. A further candidate as a TN receptor is the receptor tyrosine phosphatase β , the external domain of which is the TN binding proteoglycan called phosphacan (Barnea *et al.*, 1994). It has been proposed that the adhesive functions of TN act through the integrins and the anti-adhesive functions are through the proteoglycans/annexin II.

The presence of many types of receptor which can bind different variants of TN may partly explain the contrary and cell specific actions of TN.

(v) The function of TN *in vivo*

In order to provide definitive answers to the questions about the fundamental functions of TN, Saga *et al.* (1992) genetically engineered mice by homologous recombination to knock out the TN gene. Their initial finding was that these TN-null mice developed normally. Superficially this may be surprising if TN has a pivotal role in tissue reorganisation. However, it is possible that other members of the TN family may be capable of substituting the function of TN-C. Subsequently further investigations have shown that "knockout" mice display developmental differences (Steindler *et al.* 1995) culminating in abnormal locomotion (Fukamauchi *et al.*, 1996).

While TN clearly plays a role in embryogenesis, its role in tumour genesis and behaviour is still under investigation. The adhesive and anti-adhesive properties of the molecule have suggested that TN may play a role in tumour invasion and metastasis. The relationship with angiogenesis (Zagzag *et al.*, 1995) and the induction of proteinases (Tremble *et al.*, 1994), are also compatible with an involvement in the metastatic process, as was reviewed in section 1.2.

1.3.7 Potential roles of tenascin in tumour detection and therapy

(i) TN expression and prognosis

The relevance of TN as a prognostic factor has been investigated in a number of solid tumour types and as with many other aspects of TN research, these studies have produced contradictory results. In studies of TN expression in colon cancer Sugawara *et al.* (1993) found that colonic cancer cells were not as invasive when surrounded by TN. The presence of TN in the sera of patients with colon cancer was also investigated; TN concentrations were found to be significantly higher in the sera of cancer patients when compared to healthy individuals (Riedl *et al.*, 1995). However, Schenk *et al.* (1995) demonstrated that elevated TN levels were present in sera of patients with increased levels of acute phase protein due to infection or inflammation. In breast cancer, Moch *et al.* (1993)

found no correlation of stromal TN expression with established prognostic factors such as nodal metastases and invasion of blood vessels; they did, however, demonstrate a link between local inflammatory response and degree of TN expression.

The link between TN and local inflammatory reactions is logical as it has been demonstrated that TN synthesis may be stimulated by cytokines, such as IL-1 and IL-4 (Rettig *et al.*, 199). This is also consistent with theories put forward by Dvorak (1986) that tumours are wounds that do not heal. As Dvorak describes, most solid tumours require stroma if they are to grow beyond a size of 1-2 mm; this stroma develops in much the same way in tumours and healing wounds. The initial stages of stroma development involve the deposition of a fibrin-fibronectin gel; this gel is then infiltrated by inflammatory cells (primary lymphocytes and macrophages) and fibroblasts. The fibrin-fibronectin gel matrix is degraded and mature matrix proteins are subsequently deposited; as described previously there is overexpression of TN in matrices in both tumours and healing wounds. Further studies in breast cancer have demonstrated that there is a link between the cellular source of TN and prognosis (Ishihara *et al.*, 1995). Their data indicated that patients whose cancer cells produced TN had a poor prognosis with increased likelihood of lymph node metastasis.

(ii) TN as a target for immunotherapy

It has been demonstrated that the larger form of TN protein is more frequently expressed in neoplastic tissue than in normal tissue (Borsi *et al.*, 1992). Antibodies are available which recognise epitopes within the alternative spliced region of TN (Balza *et al.*, 1993), thus making them specific for the larger isoform of the protein. This overexpression of a specific isoform of TN in tumours is already being exploited for both the visualisation and potential therapy of gliomas. Gliomas were one of the first sites of discovery of TN (Bourdon *et al.*, 1985) and the expression of TN is well characterised. Gliomas were also one of the first systems to be

tested with anti-TN antibodies as they are suitable for intralesional therapy, due to their lack of metastasis and good compartmentalisation (Riva *et al.*, 1992).

The monoclonal antibody BC-2 is currently being used to visualise gliomas using avidin-biotin labelling in a three step process (Leprini *et al.*, 1994). The BC-2 antibody is also being used in immunoradiotherapy of gliomas, as a means of delivering radioisotopes to gliomas (Riva *et al.*, 1994). Davico-Bonino *et al.* (1995) have also developed a bispecific monoclonal antibody, which utilises TN as the tumour targeting molecule and also recognises the CD3 activation site of lymphocytes.

1.4 Scopes and objectives of this study.

The previous section details the expression and possible functions of TN in a number of solid tumours. Tamura *et al.* (1993) had demonstrated TN was present in samples of normal ovary, but it was unknown whether TN is expressed in ovarian tumours.

This thesis examines the expression and potential roles of TN in ovarian cancer. There are three main areas of study, the results of which are presented as separate chapters:

(1) Expression of TN in ovarian tumours

A series of ovarian tumours (including malignant, borderline and benign forms) were examined and compared for the expression of TN protein and RNA. The relationship between TN expression and factors such as hormone receptors and growth factors were also investigated to explore potential regulators of TN expression *in vivo*.

(2) Expression of TN by ovarian cell lines

Established ovarian carcinoma cell lines were used alongside cultures of ovarian fibroblasts developed from the ascites of patients with ovarian cancer. The use of these cell lines enabled examination of the cell types

producing TN and a study of its regulation. It has been speculated that the synthesis of TN may be under paracrine control and a co-culture model of the fibroblast and epithelial cells was used to establish whether a paracrine loop may exist in ovarian tumours. Several growth factors and hormones were investigated as potential modulating factors.

(3) Functions of TN

TN has been implicated in processes central to the development and spread of cancer, effects having been shown on cell adhesion, migration and proliferation. Cell lines were again utilised to examine the interactions of ovarian carcinoma cells with TN. The adhesive aspects of TN function involve cellular receptors; several integrins have been shown to recognise and bind TN and the expression of integrins on the cell lines was investigated.

The results from all three areas of study are summarised in a final section, which relates the data from the ovarian cell lines to the patterns of TN expression observed in the ovarian tumours. The role of TN in ovarian cancer is also speculated upon, in relation to the data from the functional assays.

Chapter 2

Materials and Methods

2.1 Materials

Listed by technique. All chemicals from Sigma unless otherwise stated.

2.1.1 Ovarian tumours

Tumour material was collected from patients presenting with ovarian tumours at the time of their initial surgery. Pathological data detailing histology, stage and grade was available for all samples. The samples were stored in liquid nitrogen.

2.1.2 Tissue culture

(i) General culture materials

DMEM	- Gibco Life Technologies
DMSO	- BDH AnalaR
Fetal calf serum (FCS)	- Advanced Protein Products
Phosphate buffered saline (PBS)	- Ovoid
Penicillin/streptomycin	- Gibco Life Technologies
Tissue culture plastics	- Falcon
TGF- α	- Boehringer-Mannheim
FSH	- Serono Laboratories (UK) Ltd.
HCG	- Serono Laboratories (UK) Ltd.
TGF- β_1	- R & D Systems

Human recombinant EGF, TGF- α , IGF-I, IGF-II and γ IFN were reconstituted in sterile PBS and stored in appropriate aliquots at -40°C. Human recombinant TGF- β_1 was reconstituted in 4 mM HCl containing BSA (1 mg/ml) and again stored at -40°C. Progesterone and β -oestradiol were dissolved in ethanol to give stock solutions of 10^{-2} M and stored in glass bijou bottles at 4°C in the dark. Stock solutions of the gonadotrophins HCG and FSH, which had been extracted from human urine, were made up in sterile NaCl diluent solution as provided with each ampoule of hormone (5000 IU/ml and 75 IU/ml respectively). HCG was used as a substitute for LH, due to its similarity to this hormone and

increased availability

(ii) Epithelial Cell Lines

The PEO1 and PEO4 cell lines were derived from the ascites of the same patient with a poorly differentiated serous adenocarcinoma (Langdon et al, 1988). The PEO1CDDP cell line was obtained by continuous exposure of the PEO1 cell line to increasing concentrations of cisplatin.

59M, a human ovarian tumour epithelial cell line and SKOV-3, a human ovarian adenocarcinoma cell line, were obtained from the European Collection of Animal Cell Cultures (ECACC).

The breast carcinoma cell lines MDA-MB-231, T47D and ZR-75-1 were also obtained from the ECACC.

(iii) Fibroblast Cell Lines

The fibroblast cell lines were cultured from the ascites of patients with primary ovarian cancer.

2.1.3 Immunohistochemistry

All antibodies were from DAKO Ltd.

Avidin biotin complex	- DAKO Ltd
DPX synthetic mounting media	- Fisons
Xylene	- Fisons

2.1.4 Reverse transcription polymerase chain reaction (RT-PCR)

(i) RNA preparation

Tri reagent LS (for cell lines)	- Molecular Research Centre Inc.
Tri reagent TM (for tumours)	- Molecular Research Centre Inc.
Chloroform	- Fisons
Ethanol	- Rathburn
Isopropanol	- BDH AnalaR
Agarose	- Gibco BRL

(ii) RT-PCR

Reaction mixture components all from Promega

123 bp ladder	- Gibco BRL
Primers	- ICRF Oligonucleotide Synthesis Laboratory
Superscript II reverse transcriptase	- Gibco BRL
Taq polymerase	- ICRF
Thermal cycler	- Techne

(iii) Probing

$\gamma^{32}\text{P}$ ATP	- Amersham
DNA 5' end labelling system	- Promega
N ⁺ membrane	- Boehringer Mannheim
Oligonucleotides	-ICRF Oligonucleotide Synthesis Laboratory

2.1.5 ELISA

All antibodies from DAKO Ltd.

Human tenascin	- Gibco BRL
Immulon 4, 96 well trays	- Immulon

2.1.6 Functional Assays

Flexible 96 well trays	- Falcon
Hanks Balanced Salt Solution	- Gibco
48 well trays	- Costar
24 well trays with inserts	- Costar
^{51}Cr sodium chromate	- Amersham

2.1.7 Fluorocytometric analysis of integrins

Antibodies

E7P6	- donated by Dr. J. Marshall, Richard Dimbleby Department of Cancer Research, St Thomas' Hospital, London.
P1E6	- Gibco Life Technologies
LM609	- Chemicon
FITC-conjugated sheep-anti-mouse Ig	- DAKO Ltd
FACSCAN analyser	- Becton-Dickinson

2.2 Methods

2.2.1 Cell Culture

(i) Routine Culture of Cell Lines

All cell lines were routinely cultured in Dulbeccos modified Eagle Medium (DMEM) at 37°C in an atmosphere of 5% CO₂ in a humidified incubator. The media was supplemented with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml) and 10% foetal calf serum (FCS) which had been heat-inactivated by incubation at 56°C for 20 min.

(ii) Establishment of fibroblast cell lines

Vials of ascitic fluid were removed from liquid nitrogen and allowed to defrost. The tube contents were resuspended in 10 ml of prewarmed DMEM (+ 10% FCS), centrifuged at 2000 rpm for 3 min and washed twice in 5 ml of the same media. After the final wash, the cell pellet was resuspended in a further 5 ml of media. An aliquot was retained for the preparation of multispot slides, and the remainder was transferred to a 25 ml tissue culture flask with a further 5 ml of media. The fibroblast cells adhered rapidly to the plastic surface of the flask and after 2-4 h the media

could be taken off, leaving behind a pure population of fibroblasts which were refed with fresh media. After the cells had been growing for 2-4 days any remaining contaminating non-fibroblast cells were removed by differential trypsinisation, the fibroblasts detaching more rapidly than the other cell types. Once the fibroblasts were well established (after 1-2 passages) an aliquot of cells was again taken for preparation of multispot slides. To prepare multispot slides the cells were washed in serum free media, counted on a haemocytometer and resuspended at a density of 5×10^5 cells/ml. An aliquot of this suspension (20 μ l) was added to each well of a multispot slide and allowed to air-dry overnight before being fixed in acetone: methanol (50%: 50% v:v) for 10 min at room temperature. These slides were stored at -20°C until use.

(iii) Cell harvesting

Routinely, cells were grown to confluence in 75 or 175 cm² flasks and fed with fresh media every 3-4 days, before being harvested, usually once a week. The confluent flasks were washed in phosphate buffered saline (PBS), pH 7.3, to remove traces of FCS. Cells were detached from the plastic by incubation with a 1:1 solution of trypsin (0.25% [w/v] in Gibco solution A) and versene (1 mM EDTA in PBS, 0.5% [v/v] phenol red), for 5-20 min at 37°C. The trypsin was inactivated by the addition of DMEM (+ 10% FCS). If the cells formed clumps during harvesting, single cell suspensions were produced by syringing the cells through a 21.5 gauge needle.

(iv) Cryopreservation and recovery of cells from liquid nitrogen

Cells to be stored were harvested as described above and the cell suspension was centrifuged to give a cell pellet. This pellet was resuspended in ice cold freezing mixture (10% [v/v] dimethyl sulphoxide [DMSO] in newborn calf serum). Aliquots were transferred to cryotubes and stored in liquid nitrogen.

To recover cells the samples were thawed and washed with DMEM (+

10% FCS) then transferred to flasks as described in section 2.2.1(ii). Epithelial cells were allowed to attach overnight at 37°C before the media was changed to remove dead cells and traces of DMSO.

(v) Cell counting

In order to estimate cell numbers for setting up experiments, cells were counted using a haemocytometer; however for counting of cells in multiple well trays a ZF Coulter Counter was used. The medium was removed from the wells; if FCS had been present cells were washed in PBS, pH 7.3. Cells were detached from the wells by incubation with 250 µl of 1:1 trypsin/versene solution and aliquots (200µl) of the cell suspensions were added to 9.8 ml of NaCl (0.9%). Each suspension was counted in duplicate. Cell numbers per well were calculated by multiplying by the dilution factor (x25).

2.2.2 Immunohistochemistry.

(i) Tumour sections

Frozen sections of the tumours (cut on a cryostat) were incubated with 20% foetal calf serum in tris buffered saline (TBS/FCS) for 10 min at room temperature. The sections were then incubated with monoclonal mouse anti-human tenascin antibody, diluted 1:100 in TBS/FCS, for 30 min at room temperature in a moist chamber. Control sections were treated with primary antibody absorbed with excess pure TN or with TBS/FCS. After washing in TBS, sections were covered in rabbit anti-mouse biotinylated antibody diluted 1:100 in TBS/FCS, followed by avidin-biotin peroxidase complex made up in TBS; both incubations were for 30 min at room temperature, in a moist chamber, and were followed by washing in cold TBS. After the final wash, sections were treated with a 1 mg/ml solution of 3,3'-diaminobenzidine, containing 5% hydrogen peroxide, for 5 min. The sections were counterstained lightly with haematoxylin. Finally the sections were dehydrated, cleared and mounted under coverslips with DPX synthetic mounting medium. Sections were examined, and

distribution and intensity of staining assessed, by two independent readers.

(ii) Multispot slides

The multispot slides were processed in a similar manner to the tumour sections with identical incubations and washes. The primary antibodies used were monoclonal mouse anti-human fibroblast (diluted 1:100), common leucocyte antigen (diluted 1:10) and epithelial membrane antigen (diluted 1:40).

2.2.3 RNA Preparation

(i) Tumours

Frozen tumour samples (~100 mg) were dismembranated, using a Mikro-Dismembrator, to a fine powder which was then added to 2 ml of Tri-Reagent™. The extraction of RNA was carried out according to the Tri-Reagent protocol; chloroform and isopropanol were used to precipitate the RNA, which was then washed in cold ethanol. The RNA samples were dried and resuspended in RNase free water. Samples were diluted (5 µl in 1295 µl) and their absorbance at 260 and 280 nm read in a spectrophotometer. Yield and purity ratios were calculated. The standard absorption coefficient for RNA is 40.

$$\text{Yield } (\mu\text{g}/\mu\text{l}) = \frac{(\text{A } 260 \text{ nm} \times \text{dilution factor} \times \text{standard absorption coefficient})}{1000}$$

$$\text{Purity} = \frac{\text{A } 260 \text{ nm}}{\text{A } 280 \text{ nm}}$$

The acceptable limits for the purity ratio are between 1.6 and 2.

(ii) Cell Lines

Confluent flasks of cells were used to prepare RNA using Tri-Reagent^{LS}, in a protocol similar to that for tumours. Additional RNA samples for ovarian, cervical and breast carcinoma cell lines were donated by Genevieve Rabiasz.

(iii) RNA Formaldehyde agarose Gels

The integrity of the RNA was determined by electrophoresis of samples in denaturing agarose gels. Samples were incubated for 10 min at 65°C with a denaturing buffer containing formaldehyde and formamide. Ethidium bromide and loading buffer (EDTA [1 mM], glycerol [50% v:v], bromophenol blue [0.25% w:v], xylene cyanol [0.25% w:v]) were added and the samples were then run on a 1.1% agarose, 6.5% formaldehyde gel at 60V for 2h. The RNA was visualised and photographed under UV light.

2.2.4 RT-PCR

(i) Reverse Transcription.

RNA (5µg) was reverse transcribed using Superscript II reverse transcriptase in a reaction mixture (total volume 20µl) containing 1.875 mM MgCl₂, 10 mM tris, 50 mM KCl, 0.1% Triton X-100, 0.3 mM dNTPs, 20 units RNasin and 120 ng random hexamer to prime the reaction. Prior to the addition of reverse transcriptase and RNasin, the mixture containing the RNA was heated to 65°C for 5 min to remove any secondary structure. After addition of the enzyme, the sample was incubated at 42°C for 1 h. The reaction was terminated by a 5 min incubation at 95°C.

(ii)Primer design and purification.

All PCR primers were synthesised using cyanoethyl chemistry and were routinely supplied fully deprotected and dried down. The primers were purified by precipitating in sodium acetate (60 mM) and MgCl₂ (2 mM) in 60% cold ethanol (v/v).

The primers designed to amplify TN cDNA were adapted from Siri *et al.* (1991). These primers are located on the periphery of the alternatively spliced region of TN's fibronectin type III repeats. Primers were also used to amplify cDNA of the house keeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT). Details of primer sequences and their products are shown in table 2.1.

Table 2.1 PCR primer sequences

Name	Sequence	product sizes
TN 5'	AGCTTCCAAGAAACACCACTT	1921 bp
TN 3'	GGGCAAGTAGGGTTATT	1651 bp 556 bp 284 bp
HPRT 5'	CTTGCTCGAGATGTGATGAA	290 bp
HPRT 3'	GTCTGCATTGTTTTGCCAGTG	

(iii) Polymerase chain reaction

Reverse transcribed product (equivalent to 1 μ g of RNA) was added to a PCR reaction mixture (total volume 20 μ l) containing 10 pmol of each primer, 0.3 mM dNTP and 2.5 units of taq polymerase in a reaction buffer containing (1.875 mM MgCl₂, 10 mM Tris, 50 mM KCl and 0.1% Triton X-100). The samples were vortex mixed and a drop of light mineral oil was added in order to prevent evaporation of the sample. PCR was carried out for 35 cycles of 93°C, 1 min; 53°C, 1 min; 72°C, 1 min. The final cycle was 72°C for 5 min to ensure complete reaction of final products. The PCR products were run on a 1.4% agarose gel containing ethidium bromide for 90 min at 100V. The positions of the bands were noted under UV light.

2.2.5 Specific Oligonucleotide Hybridisation

(i) DNA Transfer

After the positions of the bands had been noted, gels were destained by immersing in distilled water for 30 min. DNA was transferred to a nylon membrane overnight by capillary action using alkaline transfer (0.4 M NaOH). After the transfer was complete, the membrane was allowed to air-dry before being sealed in a plastic bag and stored at 4°C until required.

The gel was restained in ethidium bromide solution for 30 min and the efficiency of the transfer was checked under UV light.

(ii) Probe Labelling

Oligonucleotides were designed to distinguish the various isoforms of TN. Details of these oligonucleotides can be seen in table 2.2.

Table 2.2 Probe sequences and specificities

Name	Sequence	Products detected
oligonucleotide 5	AGGCAGACACAAGAGCAAGC	1921 bp 1651 bp 556 bp 284 bp
oligonucleotide A4	TTAGCCGTGTCTGAGGTTGG	1921 bp 1651 bp
oligonucleotide B	TGATTCCAATAGGTTGCTGG	1921 bp 1651 bp 556 bp
oligonucleotide C	GTAACGGTGGTGGATTCTGG	1921 bp

The probes were named according to the FN type III repeat in which they are located. This table illustrates the splice variants, identified by Siri *et al.* (1991), with which the oligonucleotides are capable of hybridising.

These oligonucleotides were end-labelled with $\gamma^{32}\text{P}$ ATP. Oligonucleotide (20 ng) was labelled with 1.85 mBq of $\gamma^{32}\text{P}$ ATP, in a reaction mix containing 3 units of T4 polynucleotide kinase, in 1x forward exchange buffer (provided with enzyme). The mixture was vortex mixed and incubated for 30 min at 37°C.

(iii) Hybridisation and Washing

Membranes were submerged in saline sodium citrate (SSC) at 6x the standard concentration (0.15 mM sodium chloride, 15 mM sodium citrate) and carefully rolled between two pieces of nylon gauze, to exclude air bubbles. The membranes and gauze were placed in hybridisation oven tubes and were prehybridised for 30 min at 48°C in hybridisation solution (SSC at 5x the standard concentration containing 0.1% sodium dodecyl sulphate SDS, 0.1% sodium pyrophosphate, 0.05% bovine serum albumin, 0.05% polyvinyl pyrolidone, 0.05% ficol.)

Labelled oligonucleotide was added to 25 ml of hybridisation solution. The prehybridisation solution was poured off the membranes and the label was added. Hybridisation was allowed to occur for 4h at 48°C. Filters were rinsed twice and then washed four times at 48°C for 15 min in SSC (4x standard concentration), 0.1% SDS, 0.1% sodium pyrophosphate. The filters were sealed in plastic and autoradiography was carried out at room temperature. Filters were exposed, to X-ray film, for 30 min to 48 h to obtain the optimum signal.

(iv) Reprobing

In order to reprobe the same membrane with different probes, the radiolabelled oligonucleotides were stripped from the membrane by incubating in 0.4 M NaOH for 30 min at 45°C. The membrane was then washed twice at room temperature for 10 min in 0.1 x SSC, 0.1% SDS, 0.2 M tris-HCl (pH 7.0). After stripping the membrane was autoradiographed to check no radiolabel remained.

2.2.6 Measurement of TN Secretion

(i) Production of Conditioned Medium

Cells were plated onto tissue culture flasks or trays at high density in DMEM (+ 10% FCS) and left overnight to allow attachment to the plastic. The serum-containing medium was then removed and the cells were washed in PBS before the addition of serum-free medium, phenol red-

free DMEM containing HITS (10 nM hydrocortisone, 5 µg/ml insulin, 10 µg/ml transferrin and 30 nM sodium selenite). After a wash-out period (~12h) fresh media were added and the cells incubated for 48h. After this time the conditioned medium (CM) was collected and centrifuged in polypropylene tubes at 2000 rpm to remove dead cells and other debris. The cells were then harvested and counted on either a haemocytometer or the coulter counter as described previously. The media were immediately analysed for TN by ELISA as described below.

(ii) Enzyme linked immunosorbant assay (ELISA)

96 well plastic plates (Immulon 4) were coated overnight with purified TN diluted in PBS (200µl) to give a range of 0.5 - 16 ng per well for generation of a standard curve. Each sample of CM (200µl), was also added to wells for overnight incubation. CM was assessed neat and diluted 1:2 with PBS. Following the overnight incubation the plates were thoroughly washed with PBS containing 0.5% tween-20 (PBS-T). The plate was then incubated for 2h at 37°C with mouse anti-human TN monoclonal antibody at a 1:160 dilution in PBS-T while replicate wells were left in PBS-T as a measure of background binding. The plates were then washed again in PBS-T and incubated with rabbit anti-mouse Ig diluted 1:1000 in PBS-T for 1h at 37°C before a final washing in PBS-T. 50 mg of orthophenylenediamine (OPD) was dissolved in 100 ml of substrate buffer (0.71 g anhydrous Na₂HPO₄, 0.5 g citric acid, pH 5.0). Immediately prior to use, 20µl of hydrogen peroxide was added. OPD solution (200 µl) was added to each well and the plate was incubated for 10 min at room temperature in the dark. The reaction was terminated by the addition of 50 µl of 0.5 M sulphuric acid and the optical density of each well was measured on a spectrophotometer at a wavelength of 492 nm.

2.2.7 Modulation of TN secretion

(i) Co-culturing experiments

Tissue culture inserts with 8µm diameter pores were used to co-culture fibroblast and epithelial cells. These inserts keep the cells physically separate but allow diffusion of potential paracrine factors. The cells to be co-cultured were harvested by trypsinisation and counted on a haemocytometer. Fibroblasts (2×10^5) were added to the well and a similar number of PEO1 epithelial cells were added to the inserts (in a separate tray). The cells were allowed to attach for 24h in DMEM (+10% FCS) and incubated overnight in DMEM (+ HITS). After this time the inserts were moved to the tray containing the fibroblasts.

Quadruplicate wells were set up with 3 different cell mixtures: (i) fibroblasts with PEO1 cells in the insert, (ii) fibroblasts with empty inserts and (iii) inserts with PEO1 cells in a well containing no fibroblasts. Fresh serum-free medium was added to the well and the insert (500µl and 300µl respectively). The cells were incubated for 48h at 37°C and counted; the media were collected and assayed for TN content by ELISA.

(ii) Addition of growth factors

Cells were harvested and plated out in serum-containing medium, in 24 well trays (2.5×10^5 cells per well). After overnight attachment the cells were washed, changed to serum-free conditions (DMEM + HITS) and incubated overnight.

The growth factors, hormones and cytokines whose effects were to be tested were made up to a single concentration in the serum-free media. These concentrations had previously been determined to produce a biological response in ovarian systems.

The factors were added to the cells in a total volume of 500µl of medium. The cells were incubated for 48h, counted and the medium assayed for TN content by ELISA.

Further experiments investigated selected factors over a range of different concentrations to examine whether there was a dose response effect on



TN secretion. The cells were incubated for 48h and TN secretion was assayed by ELISA.

2.2.8 Functional Assays

(i) Adhesion Assay

Non-sterile 96 well trays were coated overnight, at 4°C (or for 2h at 37°C) with extracellular matrix proteins at concentrations ranging from 1 to 40µgml⁻¹ in PBS. All proteins were plated into quadruplicate wells. To take into account non-specific binding to protein 1% BSA solution (in PBS) was used as a control. After coating the plates were washed in PBS. Non-specific adherence to plastic was blocked by incubation for 90 min in 0.1% BSA solution.

Cells were harvested in a small amount of trypsin to produce a single cell population, and labelled with chromium (⁵¹Cr) as described by Brunner *et al.* (1976). The cells were centrifuged at 2000 rpm and resuspended in 200 µl of DMEM (+ 10 % FCS). An isotonic solution of ⁵¹Cr sodium chromate (90 mCi) was added to the cell suspension and incubated for 1h at 37°C, with occasional mixing. The cells were centrifuged and washed 3 times in serum-free media to remove traces of ECM proteins and serum. An aliquot of cells was counted on a haemocytometer. Cells were then diluted to 3 x10⁵ per ml. Aliquots (50 µl) were added to each well and to counting tubes as the "input" count.

After incubation at 37°C for 2h, plates were washed twice by gently submerging the plate in PBS supplemented by cations (1 mM Ca/0.5 mM Mg). Plates were then cut into individual wells and counted in a gamma counter.

% adhesion was calculated as described by Marshall *et al.* (1995)

$$\left(\frac{\text{mean count} - \text{mean BSA count}}{\text{mean input count}} \right) \times 100$$

% standard deviation was calculated as

$$\left(\frac{\text{s.d absolute}}{\text{mean absolute}} \right) \times \% \text{ adhesion}$$

(ii) Spreading Assay

Sterile 48 well trays were coated with ECM proteins in duplicate wells as described in 2.8(i). Cells were harvested to give a single cell population and washed 3 times in serum-free media. An aliquot was counted and the cells were diluted to 1.5×10^5 per ml. 200 μ l were added to each well and the tray was incubated at 37°C for 2h. The cells were fixed in 10% formalin, examined and photographed under phase contrast microscopy.

(iii) Migration Assay

Migration assays were performed in 8 μ m-diameter pore size Transwell chambers in a method adapted from Mould *et al.* (1994). The undersurface of the polycarbonate membrane was coated with various concentrations of ECM proteins by sitting the insert in the relevant protein solution for 1 h at 37°C. The protein solution was removed and the membranes were washed in PBS. Cells were harvested to produce a single cell suspension of 4×10^5 cells/ml in serum-free DMEM containing 1% BSA. Aliquots of the cell suspension (1×10^5) were added to the upper chamber of the Transwell and 500 μ l of the same media was added to the lower chamber. The cells were incubated at 37°C for 48 h in a humidified incubator and then cells were either fixed in 10% formalin and stained with 0.1 % crystal violet, or trypsinised (with equal volumes of trypsin on each side of the membrane) and counted on a coulter counter.

With the transwells stained, the cells on the upper surface of the membrane were removed using a cotton bud. The under surface of the membrane was examined under a microscope and 3 random fields were counted using a graticule eyepiece. Migration was expressed as cells per high powered microscope field.

The trypsinisation allowed cells on both sides of the membrane to be counted and migration was expressed as the percentage of cells which had passed through the membrane

2.2.9 Fluorocytometric analysis of integrin receptors.

Cells were harvested using trypsin, spun at 2000 rpm and incubated with fresh DMEM (+ 10% FCS) for 30 min at 37°C to allow them to recover from the harvesting process. Approximately 5×10^5 cells were required for each antibody to be used and the aliquots of cells were washed once in ice-cold PBS followed by ice-cold PBS containing 1% FCS (v/v) (PBS/FCS). The cells were incubated for 30 min with primary antibody diluted in PBS/FCS. As a negative control an aliquot of cells was incubated with PBS/FCS. After washing in PBS/FCS, 100 μ l of FITC-conjugated sheep-anti-mouse immunoglobulin (ShAM/FITC) diluted 1:20 in PBS/FCS was added to each tube. The cells were incubated on ice for 30-60 min, washed twice in cold PBS and resuspended in PBS (1 ml). The suspension was analysed on a flow cytometer, which measured the green fluorescence produced by the FITC-conjugated secondary antibody.

Chapter 3

Tenascin expression in primary ovarian tumours.

Chapter 3

Tenascin expression in primary ovarian tumours.

TN has been found to be expressed in the stroma of many solid tumours and also in some normal tissues, including ovary, but has not previously been studied in ovarian tumours. This is the first study to investigate the expression of TN, both at the level of protein and RNA, in a series of malignant, borderline and benign ovarian tumours.

3.1 Protein expression

The distribution of TN protein in sections of ovarian tumours was examined by immunohistochemistry.

3.1.1 Optimal conditions for immunohistochemistry

The expression and distribution of TN in ovarian tumours was defined by use of the mouse anti-human TN monoclonal antibody obtained from DAKO. This antibody was raised against TN secreted by the U251 glioma cell line which expresses both large and small isoforms of TN and is reported not to cross react with fibronectin or EGF; this was verified by ELISA (as described in section 4.2.1). Initial experiments using paraffin fixed tumours failed to produce any staining; frozen sections of human ovarian tumours were used in subsequent studies. The optimal antibody concentration was determined to be a 1:50 dilution of the stock antibody; when compared with 1:100 and 1:25 dilutions of the antibody, this concentration provided the most economical use of the antibody whilst saturating the antigen.

For all samples a control section without primary antibody showed absence of staining. As an additional control, in a smaller number of samples, the specificity of the antibody was verified by addition of pure TN to the primary antibody, which totally abolished staining.

3.1.2 Tenascin staining distribution

The presence of TN was investigated by immunohistochemistry in 50 primary ovarian tumours (32 malignant, 7 borderline and 11 benign) and 1 normal ovary. Complete details of these tumours are shown in table 3.1

TN staining was observed in 44 of the 50 tumours at variable levels (table 3.1). Whilst all staining was in the extracellular space, the staining pattern could be classified as either focal or diffuse.

Table 3.1 a Histology and TN staining of borderline and benign tumours

Sample code	Histology	Staining pattern	Stromal intensity
Borderline			
032	serous	stromal	+
054	mucinous	stromal	+
081	mucinous	negative	-
102	mucinous	negative	-
125	mucinous	stromal	+
131	mucinous	negative	-
161	serous	stromal	++
Benign			
004	thecofibroma	focal	-
013	fibroma	focal	-
053	mucinous	focal	-
078	cystadenoma	focal	-
090	fibroma	negative	-
100	mucinous	focal	-
138	mixed	stromal	+
147	fibroma	focal	-
151	teratoma	focal	-
166	mucinous	focal	-
168	fibroma	negative	-

This table shows the histology, TN staining pattern and intensity of stromal staining in benign and borderline ovarian tumours. TN staining is classified as described for table 3.1 b.

Table 3.1 b Histopathology and tenascin staining of malignant ovarian tumours

Sample code	Histology	Stage	Grade	Staining pattern	stromal intensity
002	serous	III	moderate	stromal	+++
006	endometrioid	nk	moderate	negative	-
008	serous	III	poor	stromal	+
014	endometrioid	III	poor	stromal	++
015	endometrioid	IV	poor	stromal	+
019	serous	III	poor	stromal	+++
020	mnt	II	variable	focal	-
021	serous	III	poor	stromal	++
069	serous	III	moderate	stromal	+++
072	serous	IV	poor	stromal	++
077	clear cell	III	moderate	stromal	++
084	mucinous	III	moderate	stromal	++
088	endometrioid	IIc	moderate	stromal	+
091	serous	III	moderate	stromal	++
093	mnt	III	poor	stromal	+
095	endometrioid	Ic	poor	stromal	+++
096	endometrioid	III	moderate	focal	-

098	serous	nk	poor	focal	-
104	clear cell	Ia	moderate	focal	-
109	serous	III	poor	stromal	+
117	serous	III	poor	stromal	+
122	endometrioid	Ia	moderate	stromal	++
124	clear cell	Ia	poor	stromal	++
132	endometrioid	Ia	well	stromal	+++
134	endometrioid	Ic	poor	focal	-
137	mucinous	I	well	stromal	+
148	serous	III	poor	stromal	+
149	endometrioid	III	poor	stromal	++
162	serous	nk	nk	stromal	+
171	endometrioid	nk	poor	stromal	+++
178	endometrioid	III	nk	stromal	++
183	serous	nk	poor	stromal	+

This table shows the histological type, clinical stage and grade of the malignant ovarian tumours along with their TN staining pattern and the intensity of stromal staining. Stage and grade were classified according to the FIGO classifications, nk indicates that this data was not available for this sample. TN staining pattern was defined as stromal, negative or focal. The stromal intensity was classed as strong (+++), moderate (++) , weak (+) or negative (-).

In the section of normal ovary the observed staining was limited to a fine line around the smooth muscle cells of blood vessels with negligible reaction in the surrounding ovarian stroma (figure 3.1). Where the TN staining in tumour sections was classified as focal the immunoreactivity was confined to structures such as blood vessels, as in the normal ovary (illustrated in figure 3.2).

In sections demonstrating diffuse staining, in addition to the perivascular staining, TN expression was observed throughout large regions of the stroma (figure 3.3).

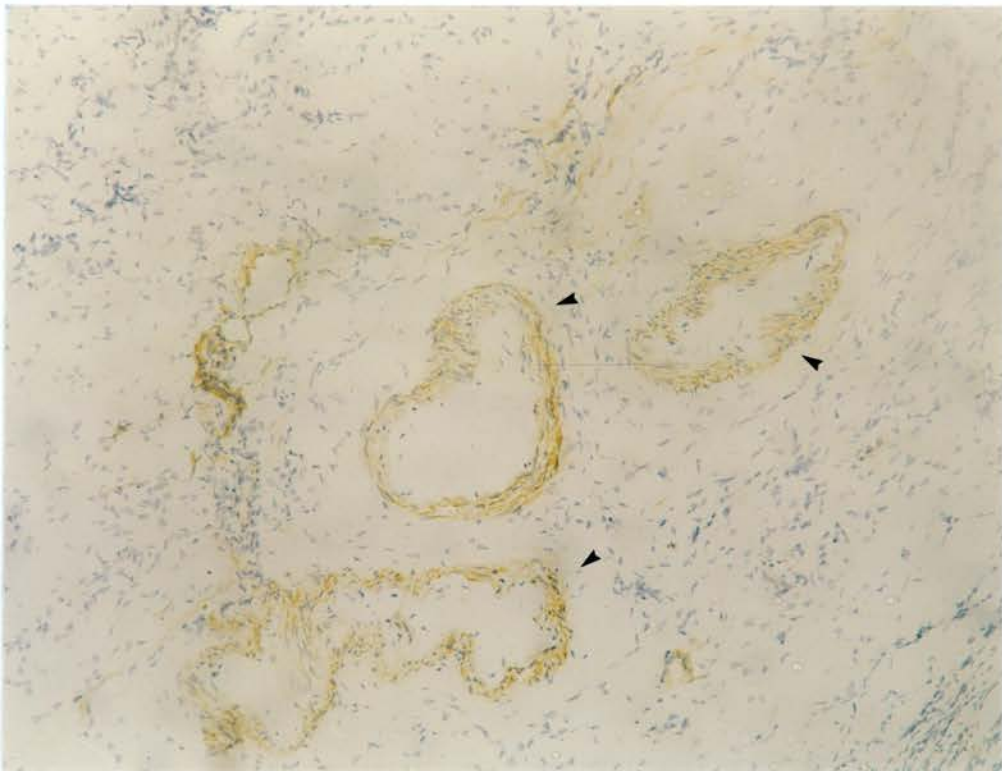


Figure 3.1 Normal ovary

Frozen section of normal ovary immunohistochemically stained with anti-human TN antibody. (Magnification= x125). Arrows indicate specific immunostaining in blood vessels

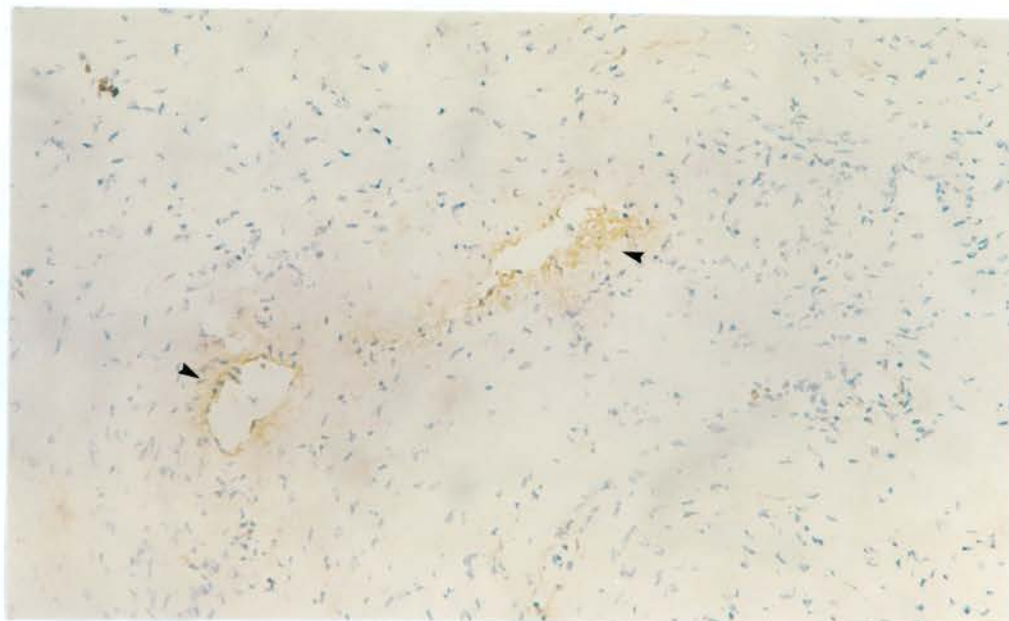


Figure 3.2 Benign tumour

Frozen section of benign tumour immunohistochemically stained with anti-human TN antibody. (Magnification= x125). Arrows indicate specific immunostaining in blood vessels

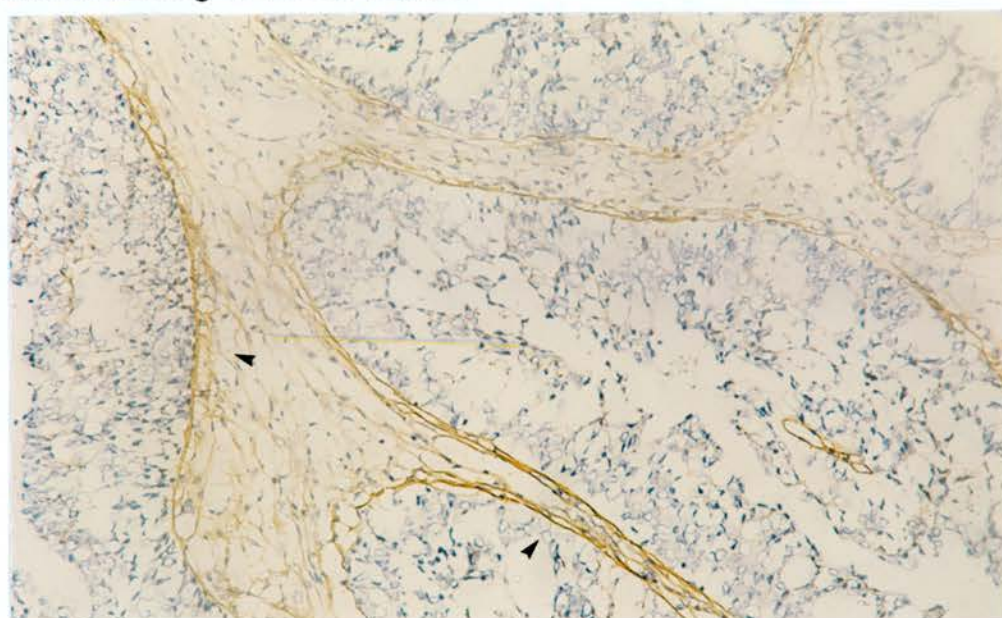


Figure 3.3 Malignant tumour

Frozen section of malignant tumour immunohistochemically stained with anti-human TN antibody. (Magnification= x125). Arrows indicate specific immunostaining

The intensity of such staining was classified as strong, moderate or weak. Heterogeneity was observed within sections of individual tumours and where it was observed the score allocated was based upon a combination of area stained and intensity of the reaction. Sections scored as strongly staining had high intensity staining over 50 to 100 % of their stroma. Moderately staining sections demonstrated a lower intensity of staining over 50 to 100% their stroma or higher intensity staining over 25 to 50%. Weakly staining tumours either demonstrated a low intensity of staining over their stroma, or only showed staining at epithelial mesenchymal junctions (less than 25%). The incidence and intensity of diffuse stromal staining is shown in table 3.2.

Table 3.2 TN Staining in Ovarian Tumours.

a) Incidence of Stromal Staining.

		Stromal Staining	
		negative	positive
Benign	(n=11)	10	1
Borderline	(n=7)	3	4
Malignant	(n=32)	6	26

b) Intensity of Stained Stroma

		Staining Intensity			
		negative	weak	moderate	strong
Benign	(n=11)	10	0	1	0
Borderline	(n=7)	3	3	1	0
Malignant	(n=32)	6	10	10	6

Both the malignant and borderline tumours showed a significantly greater incidence of diffusely stained stroma when compared to the benign tumours ($p<0.0001$ and $p=0.038$ respectively, by Fisher's exact test). There was no significant difference between malignant and borderline tumours, but numbers were small. In terms of intensity of diffuse staining, the majority of the malignant tumours showed moderate to strong staining while 3 of 4 stained borderline tumours displayed only a weak reaction. Examples of strong staining were only observed amongst the malignant tumours.

No significant associations were observed between TN expression and histology, stage or grade of malignant tumours, but numbers in the subgroups were often small. The distribution of TN staining in relation to the parameters of histological type, clinical stage and grade is shown in tables 3.3 a-c.

Table 3.3 a
Relationship between stromal TN expression and histological type

	Stromal Staining			
	strong	moderate	weak	negative
serous	3	3	6	1
endometrioid	3	4	2	3
clear cell	0	2	0	1
mucinous/mixed mesothelial tumours	0	2	2	1

These data are not suitable for chi squared analysis, as numbers within the categories are too small. When the groups are subdivided into focal or diffuse staining and compared using Fisher's test, no significant relationships emerge (serous vs endometrioid $p=0.3$; serous vs others $p=0.13$).

Table 3.3 b

Relationship between stromal TN expression and stage

	Stromal Staining			
	strong	moderate	weak	negative
I/II	2	2	2	3
III/IV	3	8	6	2

These data were also analysed by Fishers exact test and the TN distribution showed no significant association with clinical stage (stage I/II vs stage III/IV p value =0.29).

Table 3.3c

Relationship between stromal TN expression and grade of differentiation

	Stromal Staining			
	strong	moderate	weak	negative
well	1	0	1	0
moderate	1	5	1	3
poor	4	4	7	2

Again data was analysed by Fisher's exact test and no significant associations were observed (moderate vs poor p=0.7)

Omental metastases were available from 7 of the malignant tumours; all were positive and showed a similar pattern of staining to their counterpart primary tumour with strong stromal staining around the nests of tumour cells (figure 3.4).

Figure 3.4a Tumour

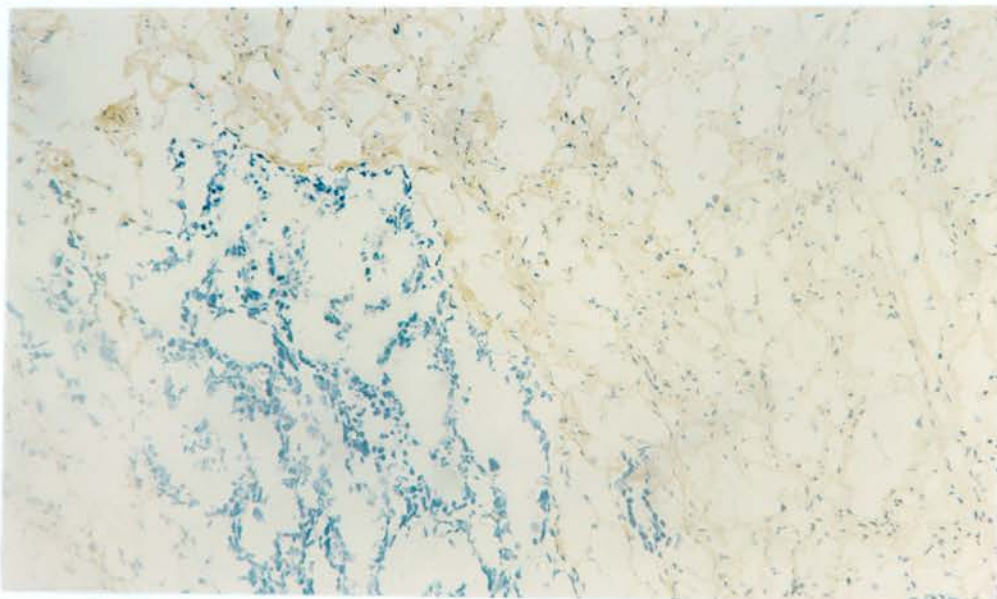
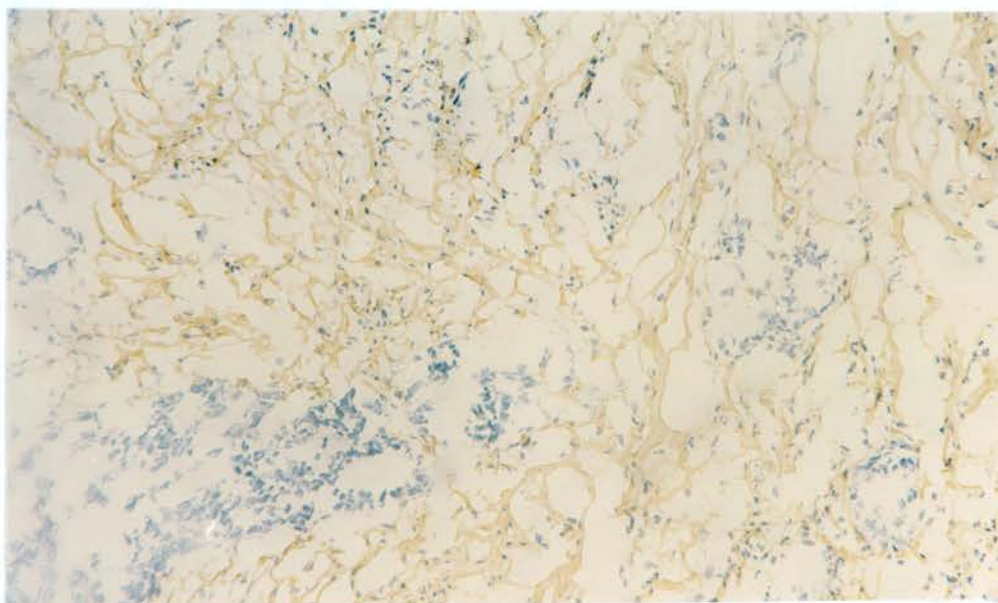


Figure 3.4b Omentum



Frozen section of paired tumour and omentum immunohistochemically stained with anti-human TN antibody. (Magnification= x125)

3.2 Tenascin RNA Expression in Primary Ovarian Tumours

In other tumour types TN RNA has been examined using northern blotting techniques (Borsi *et al.*, 1992; Ibrahim *et al.*, 1993). Siri *et al.* (1991) developed PCR primers within the region of TN's FN type III repeats. These primers are capable of detecting multiple splice forms of TN RNA, and the advantage of using RT-PCR is that smaller quantities of RNA are required than in northern blotting.

3.2.1 Preparation of RNA samples and validation of results

RNA was prepared from 12 malignant tumours (5 serous and 7 endometrioid) and 6 benign tumours (2 mucinous cystadenomas, 3 fibromas and 1 teratoma). These tumours were all samples which had not previously been through freeze-thaw cycles, to decrease the risk of the RNA being degraded. The RNA integrity was examined on an RNA formaldehyde gel, an example of which can be seen in figure 4.5. The RNA was reverse-transcribed to DNA and amplified using PCR. After electrophoresis of the PCR mixture, multiple bands could be visualised. These included bands with apparent molecular weights at 284, 556, 1651 and 1924 bp, corresponding to products identified and sequenced by Siri *et al.* (1991). In addition to these bands other products were visible, notably at approximately 490 bp and 750 bp. In all samples tested the smallest band (284 bp) was detected and its presence was used as an internal positive control. As an additional control samples were amplified with primers to the house keeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT); absence of this RNA usually indicated degradation of the sample which was consequently discarded.

In order to help characterise the bands the DNA was transferred to membranes using alkaline transfer and probed with a series of internal oligonucleotides designed to distinguish the different isoforms. The position of these oligonucleotides in the TN monomer is illustrated in figure 3.5. Probing with oligonucleotide 5 confirmed the authenticity of the products, while the oligonucleotides A4, B and C distinguished the PCR products by identifying which fibronectin type III repeats they contained. Oligonucleotide B hybridises with all bands except the 284 bp form, which does not contain FN type III repeat B, while oligonucleotides A4 and C only hybridise with isoforms which contain FN type III repeats A4 and C respectively.

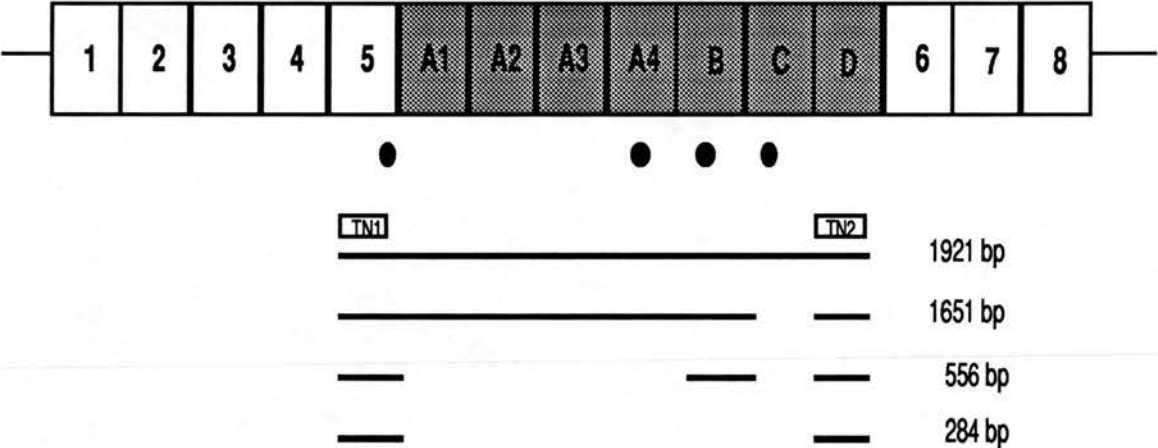


Figure 3.5

This schematic diagram of the FN type III repeats of TN indicates the region of alternative splicing (filled boxes) and shows the position of the PCR primers TN1 and TN2 on the periphery of this region. The PCR products previously identified by Siri *et al.* (1991) are shown with their length in base pairs. The position of the oligonucleotides used to probe the PCR products is also indicated (•). Within the text the probes are referred to as oligonucleotide 5, A4, B and C according to the FN type III repeat in which they are found.

3.2.2 Incidence of different Tenascin RNA isoforms in ovarian tumours.

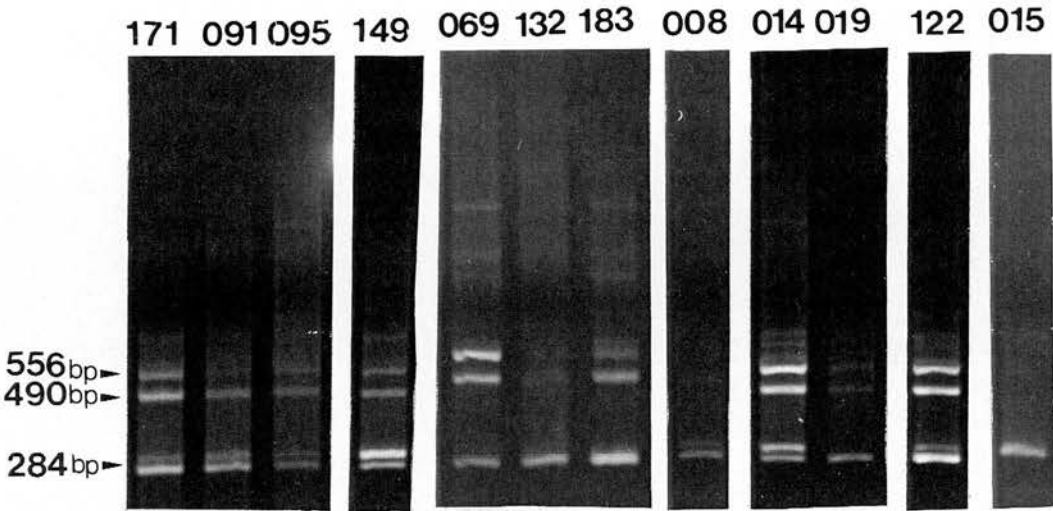
Examples of gels showing TN PCR products can be seen in figure 3.6. Whilst multiple RNA isoforms were detected in all the tumours investigated, incidence of the different molecular weight bands varied between tumour types. The 284 bp band appeared to be a constant feature of all the tumour samples, while the relative proportions of the other bands varied between tumours. The bands of ~490 and 556 bp were present at a higher intensity than the lower molecular weight form in 9 of the 12 malignant samples, the exceptions being HOV 008, HOV 015 and HOV 132. Of the benign tumours the only sample with a relatively high intensity of the ~490 and 556 bp bands was HOV 151. The sample of normal ovary appeared to express the same pattern of isoforms as the benign samples. The occasional appearance of double bands at the 284 bp position is likely to be artefactual as the expression of the doublet was not consistent within duplicate samples.

The gels shown in figure 3.6 were transferred to membranes and probed with the oligonucleotides designed to distinguish the different RNA isoforms. Examples of these probed membranes can be seen in figures 3.7 to 3.10. Figure 3.7 demonstrates that all the sizes of bands observed on the gels were detected by the specific internal oligonucleotide, providing evidence for the specificity of the reaction. From the PCR gels and the membranes probed with oligonucleotide B (an example of which is shown in figure 3.8) it is apparent that two of the RNA species (~490 and 556 bp) show a distribution which is limited more to malignant ovarian tumours than benign. The presence of these bands as the predominant RNA species is noted in table 3.4. Whilst the intensity of bands can not be compared directly between tumours, because of the non-quantitative nature of the PCR reaction, the relative intensity of the bands within each tumour sample may be compared across samples.

In 9/12 (75%) malignant tumours there was increased intensity of the ~490 and 556 bp bands, compared to the 284 bp band. In only 1/6 (17%) benign tumours were these two bands present at relatively high intensity; in the majority of benign tumours and the normal ovary sample these bands were either totally absent or greatly reduced compared to the 284 bp species. The benign tumour which did contain the ~490 and 556 bp bands was the teratoma. There is a statistically significant difference between the malignant and benign expression patterns (75% versus 17%), giving a p value of 0.043 by Fisher's exact test.

The number of tumours analysed by RT-PCR was small (18) and no correlation was observed between the isoforms expressed and the pattern and level of TN immunostaining.

Malignant tumours



Benign tumours

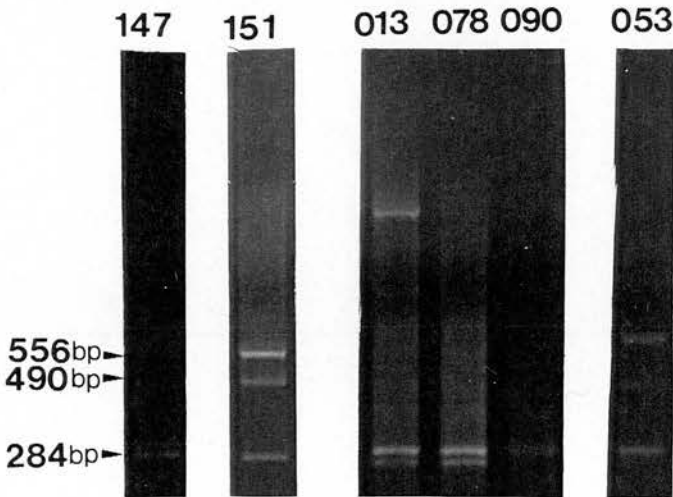


Figure 3.6 RT-PCR analysis of TN in ovarian tumours

1.4 % agarose gels containing TN PCR products. This figure contains combined data from multiple PCR analyses, all tumours tested in this study are included in the figure. The lowest molecular weight splice variant can be observed in all samples. The bands of ~490 and 556 bp are present at relatively high intensity in the malignant tumours, with the exceptions of tumours 008, 015 and 132. The only benign tumour with relatively high intensity of the ~490 and 556 bp bands is sample 151.

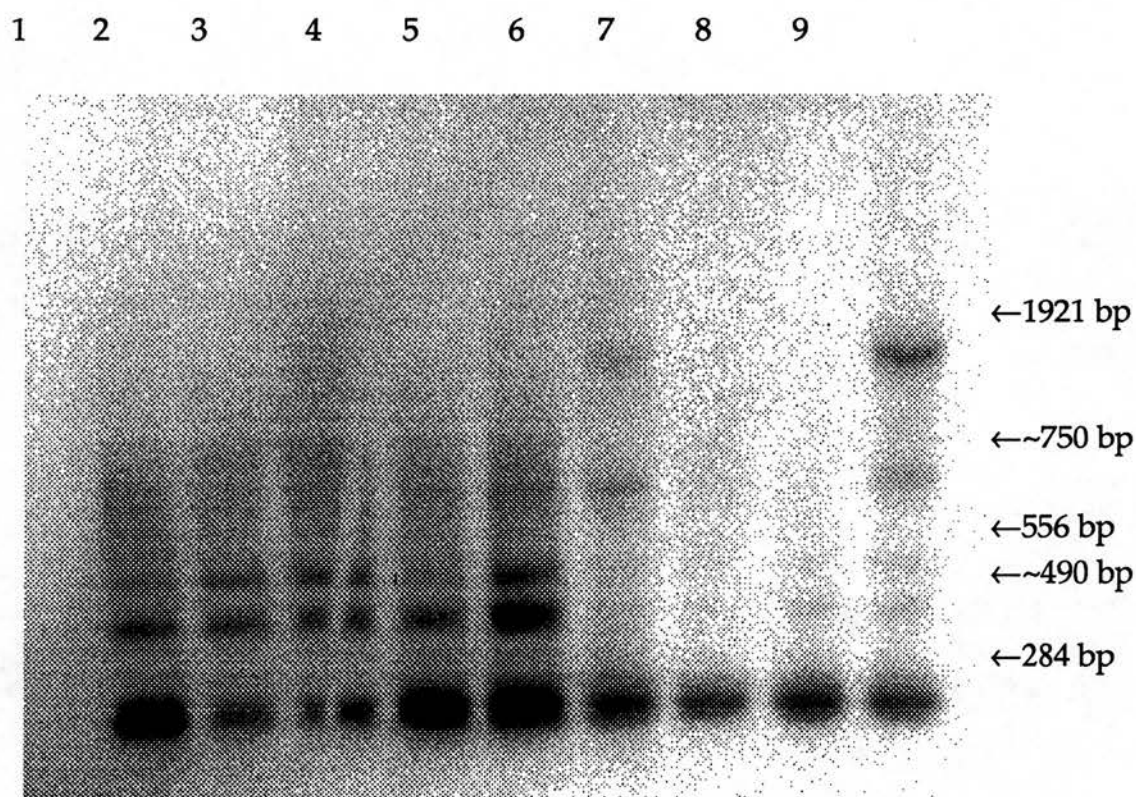


Figure 3.7 Probing of TN PCR products with oligonucleotide 5

A PCR gel containing samples of malignant and benign ovarian tumours was transferred to a nylon membrane and probed with oligonucleotide 5, which is capable of detecting all the RNA isoforms amplified by the PCR primers. The arrow indicates the 284 bp band. Lanes 1-5 contain malignant tumours (HOV 183, HOV 069, HOV 014, HOV 091, HOV 171); lanes 6-8 contain benign tumours (HOV 013, HOV 147, HOV 078); lane 9 contains normal ovary. Bands are compared within each lane rather than between lanes.

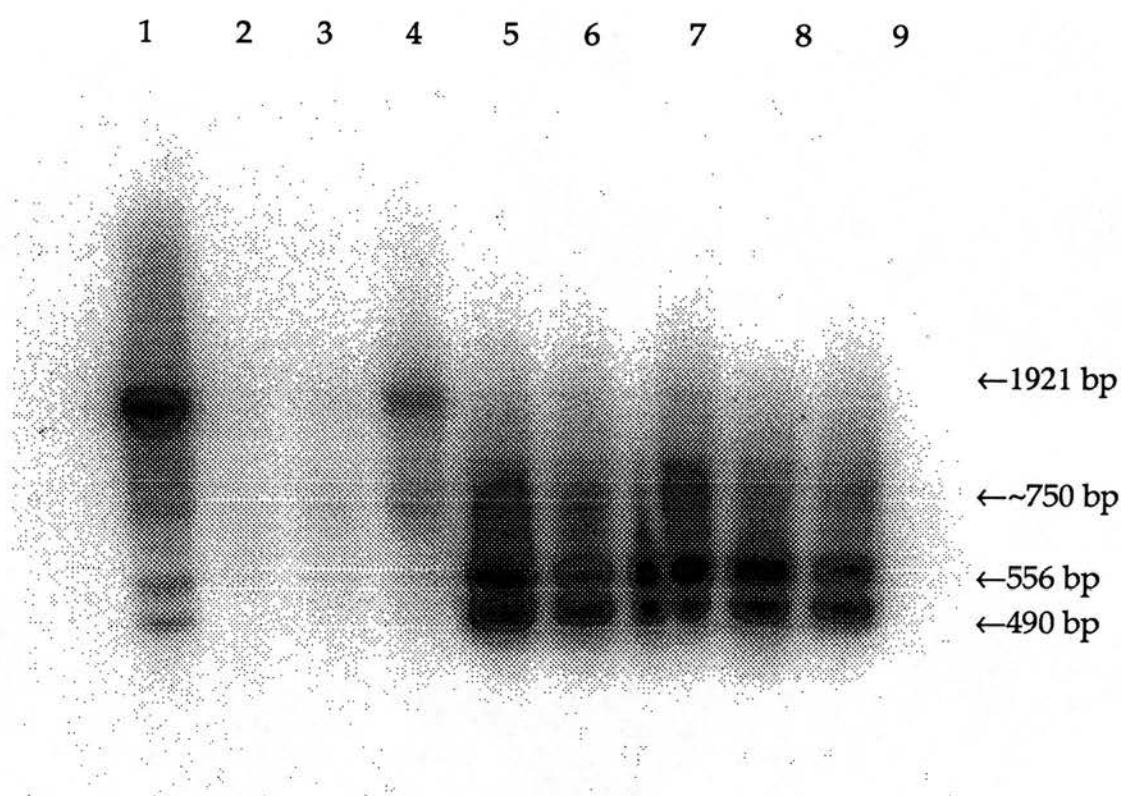


Figure 3.8 Probing of TN PCR with oligonucleotide B

Probing the membrane with oligonucleotide B detects all but the smallest isoform of TN and illustrates most clearly illustrates the difference between the malignant and benign samples. Arrows indicate the 556 and ~490 bp bands. Lane 1 contains normal ovary; lanes 2-5 contain benign tumours (HOV 078, HOV 147, HOV 013); lanes 6-9 contain malignant tumours (HOV 171, HOV 091, HOV 014, HOV 069, HOV 183).

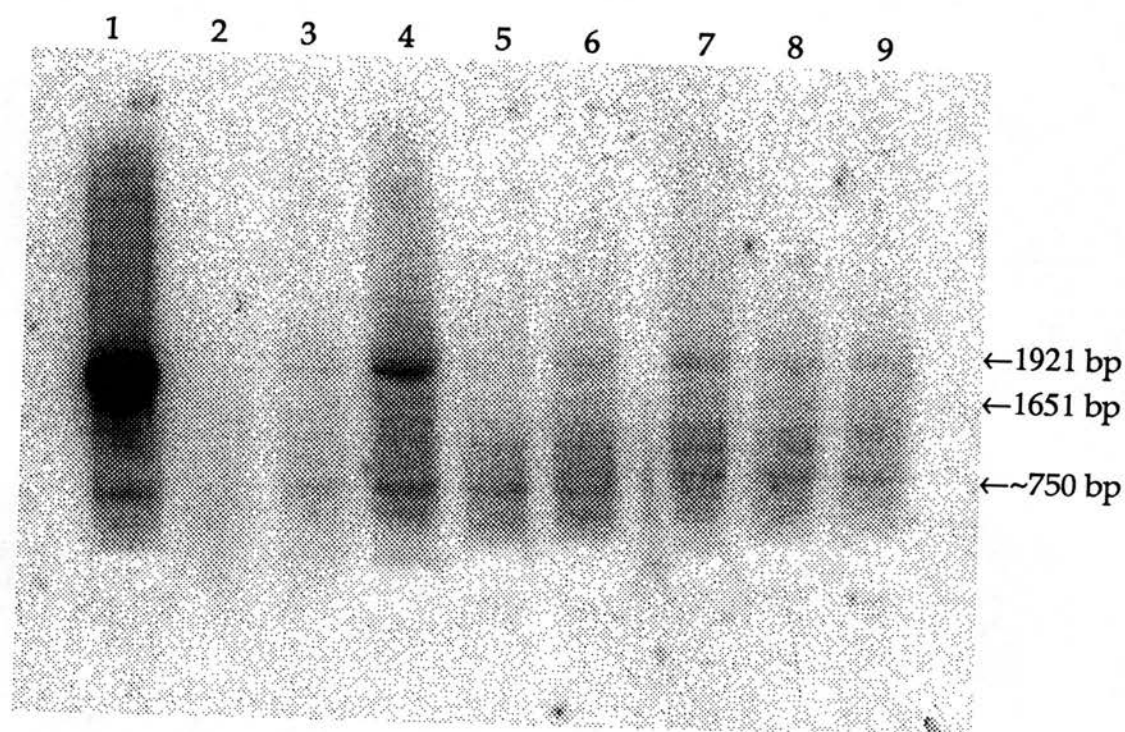


Figure 3.9 Probing of TN PCR products with oligonucleotide A4

This oligonucleotide can only detect splice variants which contain FN type III repeat A4. This membrane, was probed with this oligonucleotide after being probed and stripped twice before. Although background levels are higher, it can be seen that only higher molecular weight bands react with this probe. Lane 1 contains normal ovary; lanes 2-5 contain benign tumours (HOV 078, HOV 147, HOV 013); lanes 6-9 contain malignant tumours (HOV 171, HOV 091, HOV 014, HOV 069, HOV 183).

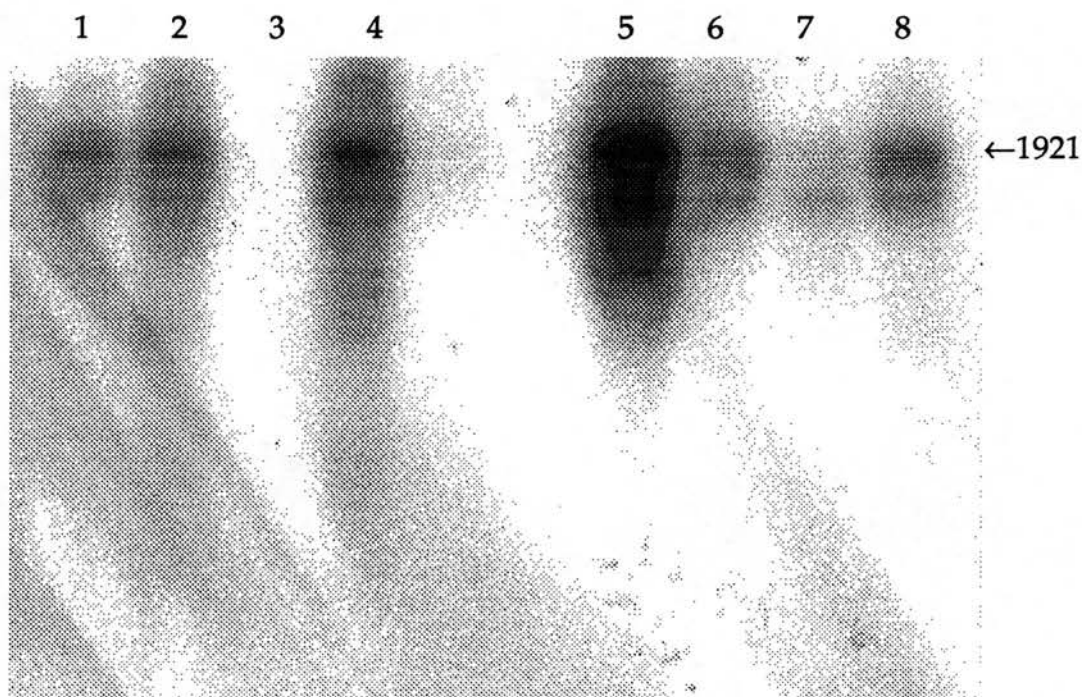


Figure 3.10 Probing of TN PCR products with oligonucleotide C

This membrane contains a different group of malignant and benign tumours to the previous three figures. Oligonucleotide C detects only isoforms containing FN type III repeat C. Lanes 1-4 contain benign samples (HOV 078, HOV 090, HOV 053, HOV 147); lanes 5-8 contain malignant tumours (HOV 183, HOV 132, HOV 069, HOV 008). One major band can be observed in most of the samples on the membrane this corresponds to the full TN transcript.

Table 3.4

Expression of TN RNA isoforms in ovarian tumours

Sample	Histology	Predominant isoform
Malignant tumours		
008	serous	284 bp
014	endometrioid	~490/556 bp
015	endometrioid	284 bp
019	serous	~490/556 bp
069	serous	~490/556 bp
091	serous	~490/556 bp
095	endometrioid	~490/556 bp
122	endometrioid	~490/556 bp
132	endometrioid	284 bp
149	endometrioid	~490/556 bp
171	endometrioid	~490/556 bp
183	serous	~490/556 bp
Benign tumours		
013	fibroma	284 bp
053	mucinous	284 bp
078	mucinous	284 bp
090	fibroma	284 bp
147	fibroma	284 bp
151	teratoma	~490/556 bp
	Normal ovary	284 bp

3.3 Association of tenascin expression with growth factors, hormones and their receptors.

In other tumour types the expression of TN has been shown to correlate with a number of other factors. The most widely reported correlation is that with the growth factor TGF- β_1 (Walker *et al.*, 1994). Vollmer *et al.* (1994) also demonstrated that hormones can effect the expression of TN. Data examining the expression of TGF- β 's, oestrogen and progesterone receptors and other growth factors was available for many of the malignant tumours examined for TN distribution. These data were investigated to identify any statistically significant correlations between TN protein expression and the factor.

3.3.1 Tenascin expression related to TGF- β mRNA expression.

A subgroup of 23 malignant tumours had previously been analysed for expression of TGF- β isoforms by RNase protection assay as described by Bartlett *et al.* (1992). These data are illustrated in table 3.5 along with the TN protein distribution for each sample. The numbers of benign and borderline tumours were too small to develop an analysis.

Sample RNA extracts were considered positive for each TGF- β isoform when a band of the appropriate length was observed in the presence of a positive transcript for control actin.

Table 3.5

Expression of TN and the TGF- β family in malignant ovarian tumours

Sample	TN intensity	TGF- β isoform expression		
		TGF- β_1	TGF- β_2	TGF- β_3
002	+++	+	+	+
006	-	+	-	+
008	+	+	-	+
014	++	+	+	+
015	+	-	-	+
019	+++	+	-	-
020	-	-	+	-
021	++	+	+	-
069	+++	+	+	+
072	++	+	+	-
077	++	+	+	+
095	+++	-	+	-
096	-	-	+	-
104	-	-	+	+
109	+	-	+	+
117	+	+	+	-
122	++	+	+	-
124	++	+	+	+
132	+++	+	+	+
134	-	+	-	-
137	+	-	+	-
148	+	-	-	-
149	++	-	+	-

This table illustrates the expression of stromal TN and TGF- β 's in a series of 23 malignant ovarian tumours. The intensity of stromal TN expression is scored as strong (+++), moderate (++), weak (+) or negative (-). TGF- β isoforms are either present (+) or absent (-).

Of the 23 tumours examined, 14 expressed TGF- β_1 mRNA, 17 expressed TGF- β_2 mRNA and 13 expressed TGF- β_3 mRNA. To examine the co-expression of TN with these TGF- β isoforms the tumours were divided into two groups; those with no/weak stromal expression of TN and those with moderate/high levels of stromal TN (table 3.6).

Analysis by Fisher's exact test showed that there was a statistically significant relationship between expression of TGF- β_1 and high levels of stromal TN expression (p value = 0.036). TGF- β_2 showed a similar trend without reaching statistical significance. TGF- β_3 did not show a significant relationship.

Table 3. 6
Relationships between stromal TN expression and the three TGF- β isoforms.

	TGF- β_1 expression		
	Positive	Negative	
High TN (++/+++)	10	2	p=0.036
Low TN (+/-)	4	7	

	TGF- β_2 expression		
	Positive	Negative	
High TN (++/+++)	11	1	p=0.069
Low TN (+/-)	6	5	

	TGF- β_3 expression		
	Positive	Negative	
High TN (++/+++)	7	5	p=1.00
Low TN (+/-)	6	5	

p-values by Fisher's exact test

3.3.2 Tenascin expression related to other parameters

Data, relating to the ovarian tumours, were available for a number of other factors. For analysis, the TN staining was again divided into those tumours expressing strong (+++/++) stromal TN and those with negative/weak stromal TN expression.

(i) Oestrogen receptor

The levels of oestrogen receptor were measured by enzyme-immunoassay by Dr Tony Hawkins in the Department of Surgery, Royal Infirmary, Edinburgh. The distribution of the receptor levels, in relation to the level of stromal TN staining is illustrated in figure 3.11.

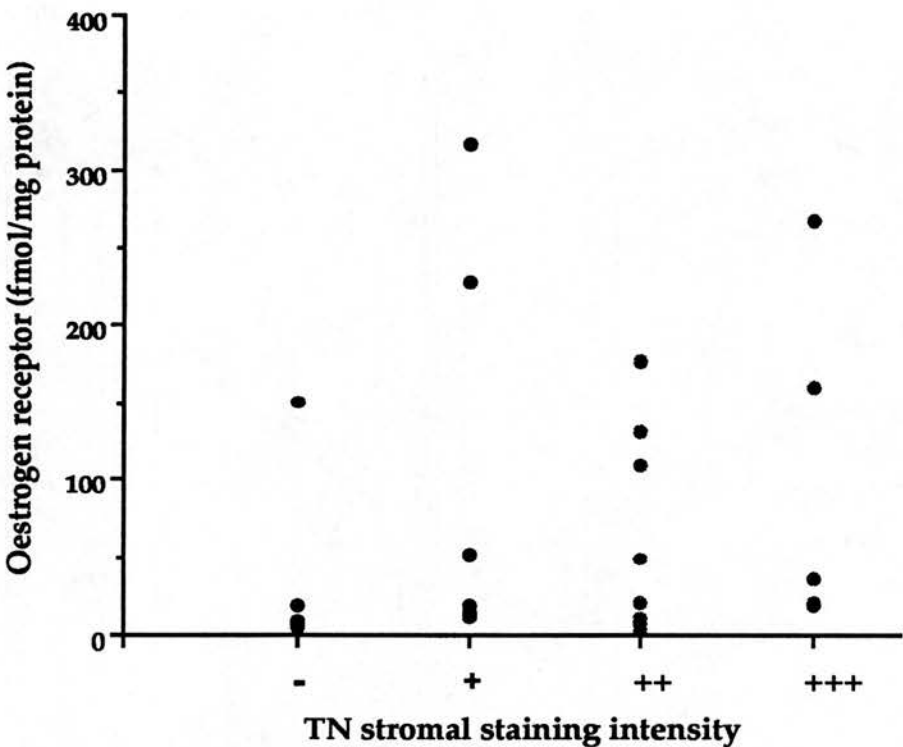


Figure 3.10 Relationship between TN expression and oestrogen receptor levels.

The points on this graph represent the level of oestrogen receptor in individual tumours, measured in fmol/mg of protein. No significant relationship was observed between the level of oestrogen receptor expressed and the degree of TN staining, as analysed by Kruskal-Wallis test (p value=0.14)

(ii) Progesterone receptor

The levels of progesterone receptor were also measured by enzyme immuno-assay by Dr. Tony Hawkins. Figure 3.12 illustrates the distribution of progesterone receptor levels in relation to TN staining

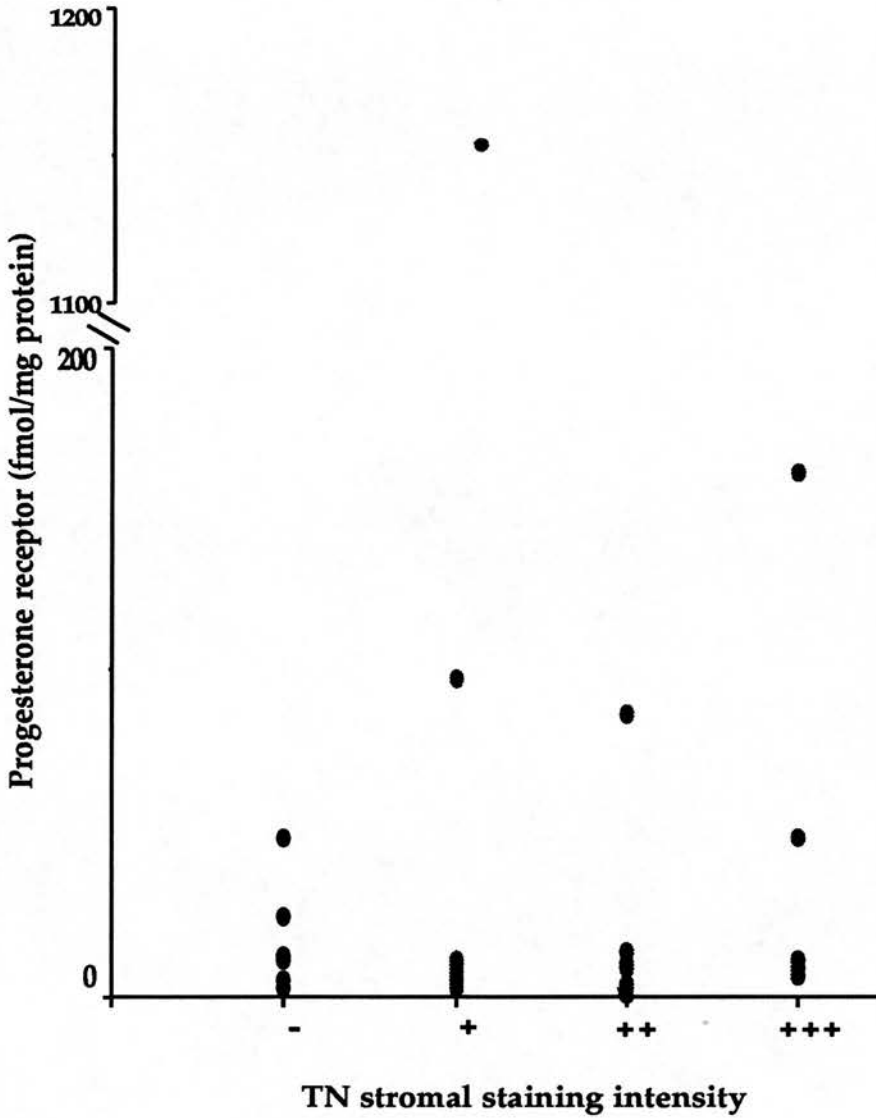


Figure 3.12 Relationship between TN expression and progesterone receptor levels.

Each point on this graph represents the level of progesterone receptor in an individual tumour, expressed in fmol/mg of protein. No significant relationship was revealed between levels of progesterone receptor and TN staining (p value=0.49 by Kruskal-Wallis test).

(iii) EGF,TGF α and EGF receptor mRNA

The presence of mRNA for EGF, TGF α and EGF receptor was measured by RT-PCR (Bartlett *et al.* 1996). Again analysis by Fisher's exact test demonstrated no statistically significant correlation with any of these factors.

Table 3.7
Relationship between TN expression and growth factors.

	EGF expression	
	Positive	Negative
High TN (++/+++)	2	8
Low TN (+/-)	4	5

p=0.35

	TGF- α expression	
	Positive	Negative
High TN (++/+++)	5	5
Low TN (+/-)	1	8

p=0.14

	EGF receptor expression	
	Positive	Negative
High TN (++/+++)	5	5
Low TN (+/-)	3	6

p=0.65

Chapter 4

Tenascin expression in ovarian cell lines

Chapter 4

Tenascin expression in ovarian cell lines

As described in the previous chapter TN is expressed in the stroma of malignant ovarian tumours at a higher level than in normal ovary or benign tumours. Previous studies of non-ovarian systems have indicated that fibroblast and some epithelial cells are capable of producing TN. To help determine which cell types produce TN, experiments were undertaken using ovarian carcinoma and fibroblast cell line models. This chapter describes the expression of TN protein and RNA in human ovarian fibroblast and carcinoma cell lines.

4.1 Characterisation of cell lines

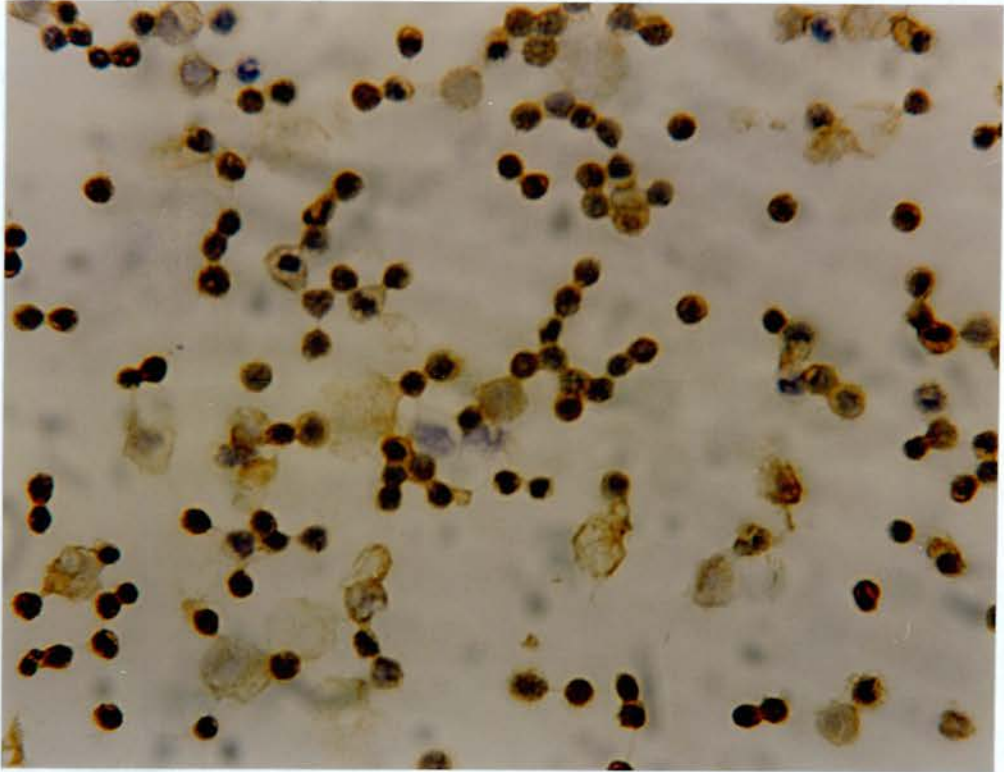
The fibroblast cells used in these experiments were derived from the ascites of patients with ovarian cancer. The PEO12 and PEO27 cells were from a patient with serous cystadenocarcinoma. The PEO9 cells were from a patient with adenocarcinoma (unclassified histology) and data was not available for the PEO13 cells. The frozen ascites samples were recovered from liquid nitrogen and transferred to tissue culture flasks; the cells were refed with fresh media after 2-4 h so that only the attached cells remained. The fibroblast cells are thus selected on the basis of their rapid attachment to tissue culture plastics. Once the cultures were established, after 1-2 passages, multispot slides were made and immunocytochemistry was used to compare the fibroblast cultures with the original ascites population. Ascites fluid from ovarian cancer patients usually contains fibroblasts, macrophages and often tumour cells. Antibodies were therefore chosen specifically to detect these cell types (leucocyte common antigen, epithelial membrane antigen and fibroblast antigen); a range of dilutions was used to first determine the optimum antibody concentration. Control spots were treated with no primary antibody

The original ascitic cell population for the cell line PEO12 consisted predominantly of leucocytes with a smaller proportion of fibroblasts and very few epithelial cells. The proportions of these cells was estimated to be 85% leucocytes, 10% fibroblasts and 5% epithelial cells. In contrast when the cells had been cultured to allow selection of the fibroblast population, few cells remained which demonstrated staining with the antibodies to common leucocyte antigen or epithelial membrane antigen. Most of the cells did stain with the fibroblast antibody. It was estimated that the final population was over 99% fibroblasts. Subsequent culturing was designed to maintain and improve upon this level of purity, by discarding cells which did not detach rapidly upon trypsinisation. The three other fibroblast cell lines (PEO9, PEO13 and PEO27) were observed in culture to consist of cells with a morphology similar to that of the PEO12 cells which stained only with the fibroblast antibody.

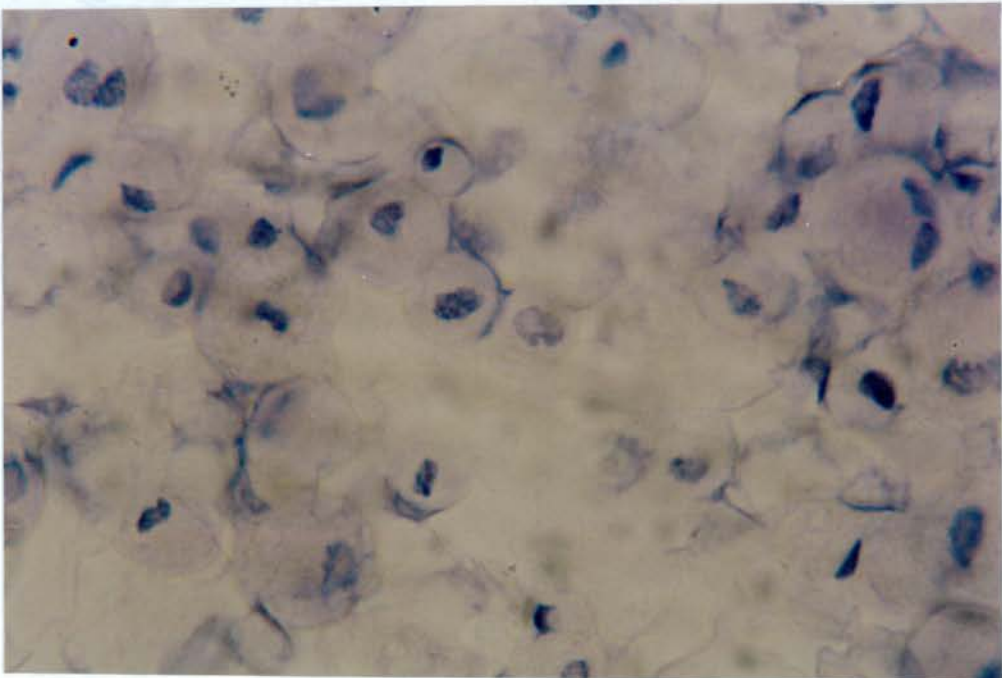
Examples of the staining observed in multispots can be seen in figures 4.1-4.3

Figure 4.1 Immunocytochemical staining of multispot slides with leucocyte common antibody.

Ascites



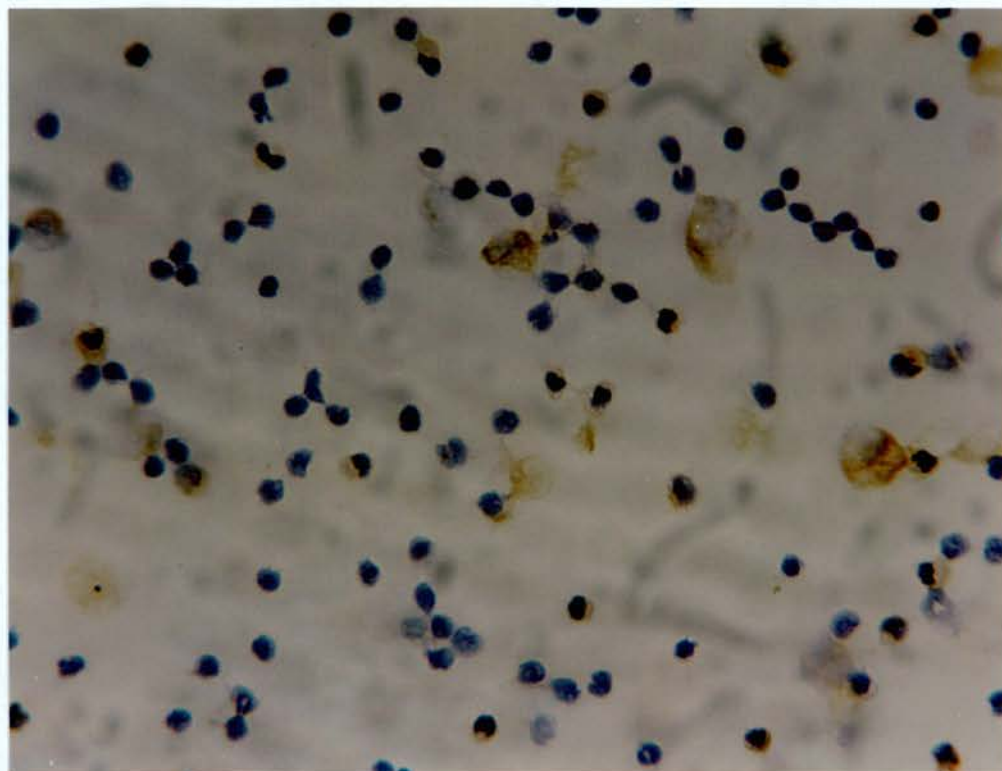
Fibroblast culture



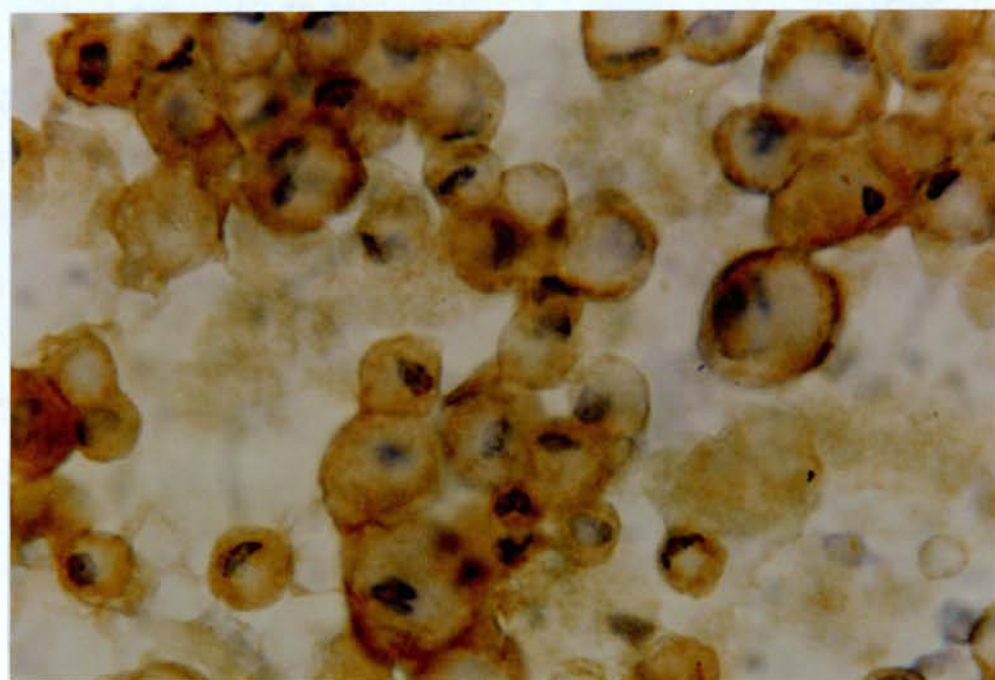
Ascites demonstrating positive staining with leucocyte common antibody (magnification = x400). Fibroblast culture demonstrating no stained cells with the same antibody (magnification = x400).

Figure 4.2 Immunocytochemical staining of multispot slides with fibroblast antibody.

Ascites



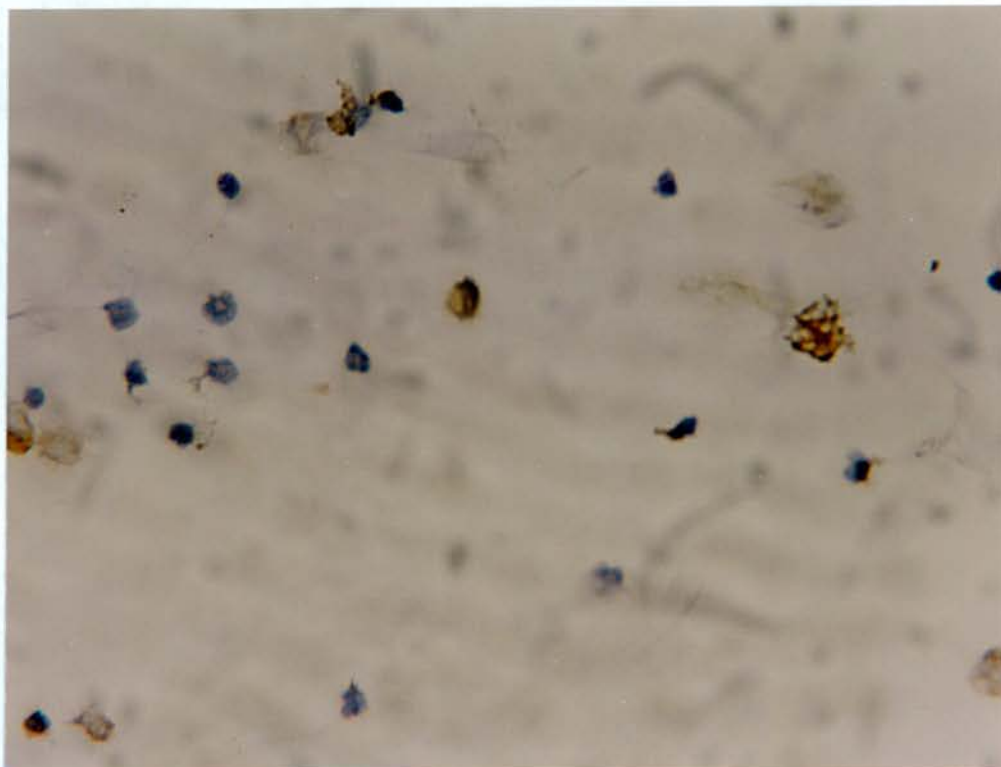
Fibroblast culture



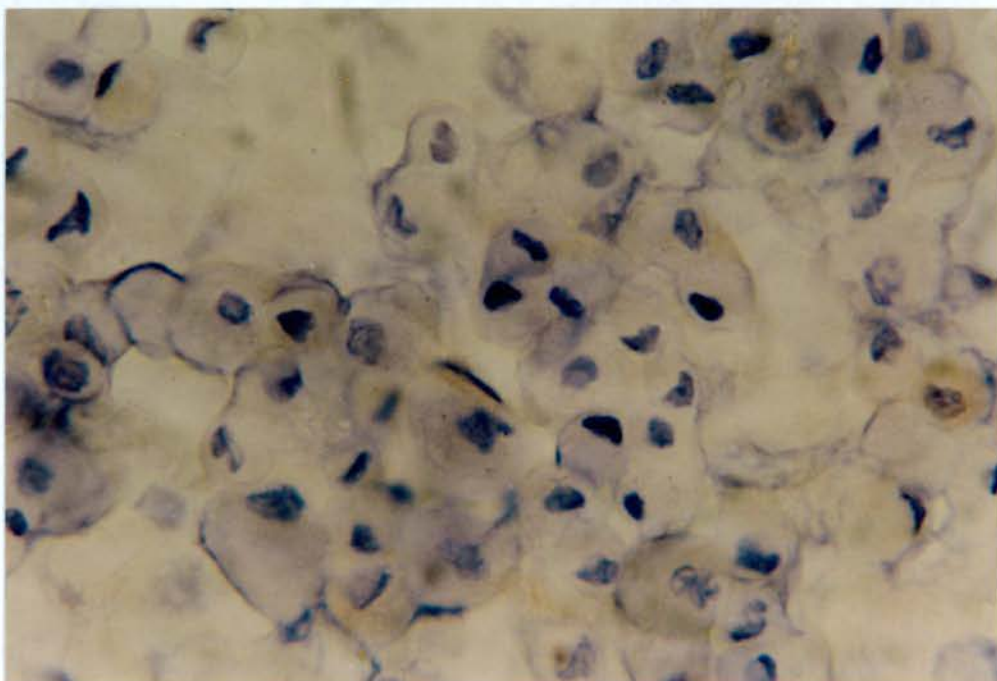
Relatively few cells on the slide containing ascites stain positively with fibroblast antibody (magnification = $\times 400$), while the fibroblast culture

Figure 4.3 Immunocytochemical staining of multispot slides with epithelial membrane antibody.

Ascites



Fibroblast culture



Relatively few cells on the slide containing ascites stain positively with epithelial membrane antibody (magnification = $\times 400$) and the fibroblast culture demonstrates no staining with the same antibody (magnification = $\times 400$).

4.2 Measurement of Tenascin by ELISA

The amount of TN secreted into the media of cultured cells was measured by an ELISA.

4.2.1 Validation of ELISA

The ELISA was optimised, before it was routinely used to measure TN content in samples.

(i) Antibody concentration

The antibody used was the mouse monoclonal anti-human TN antibody, which had previously been used for the immunohistochemical staining of the primary ovarian sections. In order to determine the optimal concentration of antibody to use in the assay, dilutions of standard TN (ranging from 0.125 to 32 ng of TN per well) were incubated with varying concentrations of the antibody, diluted in phosphate buffered saline containing tween (PBS-T). The absorbance readings from the assay were plotted and are illustrated in figure 4.4. Concentrations of antibody, ranging from 1:10 to 1:80 dilutions of the stock solution, produced parallel straight lines of absorbance versus TN content, down to the level of 0.5 ng per well. Lower concentrations of antibody (1:160 to 1:640) produced similar straight lines with a steeper gradient; however the lines of the 1:320 and 1:640 dilutions overlapped, especially at the lower limits of the assay.

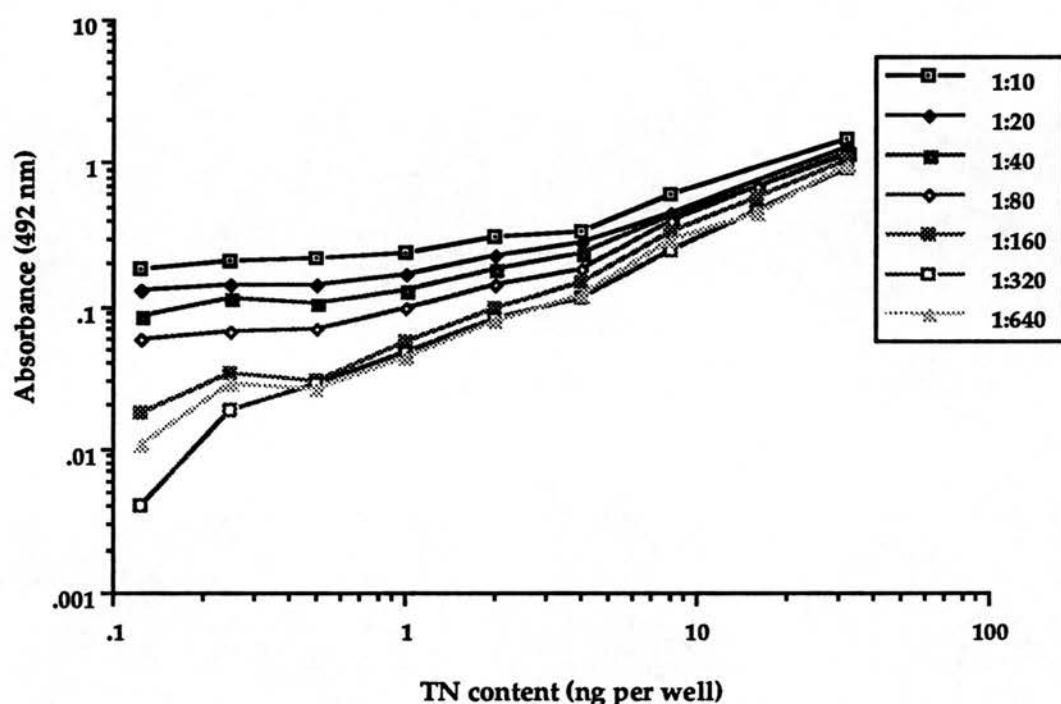


Figure 4.4 Titration of antibody concentration for ELISA

In the light of these results the concentration of antibody chosen to be used in further assays was a 1:160 dilution. This was the concentration of antibody that produced a steep gradient of separation over the range of standards used in the assay, clearly discerning different amounts of TN down to 0.5 ng per well.

(ii) Cross reactivity

The cross reactivity of the antibody was examined using fibronectin and EGF, because of TN's homology with these proteins. The antibody did not produce a colorimetric response over and above background values with fibronectin or EGF, at amounts between 15 and 2000 ng/well. This is illustrated in figure 4.5.

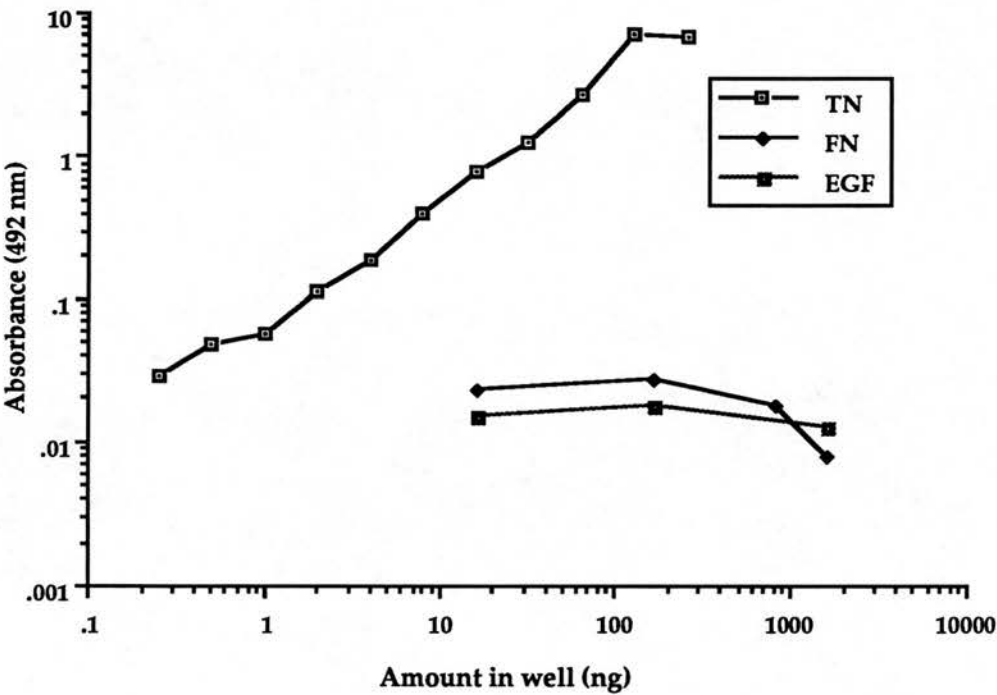


Figure 4.5 Cross reactivity of ELISA with TN, fibronectin (FN) and EGF

Initial experiments showed that media containing serum cross reacted with the antibody. A sample of media containing 10% foetal calf serum when assayed in the ELISA, appeared to contain 46 ng of TN per ml. When samples of conditioned media were corrected for this amount of TN most appeared to contain very little or no TN, whereas the same cell types cultured in serum free conditions were seen to secrete measurable levels. It therefore appears that serum contains a factor, possibly bovine TN, which reacts with the anti-human TN antibody and masks the human TN secreted into the media by the cell lines.

It was therefore decided that cells to be used in the assay should be grown in serum-free media (DMEM + HITS), which did not cross react in the ELISA. As serum has previously been shown to be a potent inducer of TN it is logical that to measure the "basal" level of TN secretion, cells should be unstimulated by serum.

It was determined whether samples could be stored frozen, prior to assaying for TN content without affecting results, by storing the samples in polypropylene tubes overnight at -40°C. The frozen samples were thawed and reassayed. Levels of TN were markedly lower in the samples which had been frozen. Conditioned media from the cell lines were therefore assayed immediately after collection.

4.2.2 Basal levels of TN secretion in cell lines

Conditioned media were collected from cell lines cultured under serum-free conditions for 48 h. These media were then assayed by ELISA (neat and diluted 1:2) to assess the amount of TN secreted by the cells.

The number of cells secreting into the media were counted and the measurements of TN secretion were corrected for cell number. This was important as the fibroblasts reached confluence with a much lower number of cells than the epithelial cell lines. Within each assay duplicate samples were measured and the secretion of TN was expressed as ng

produced in the 48 h incubation period per million cells per ml of media (ng of TN /10⁶ cells/ml/48 h).

Media from each cell line was assayed on a minimum of two separate occasions and the mean secretion of TN was calculated (table 4.1; figure 4.6). The 1:2 dilutions routinely assayed for all samples were shown to dilute as expected giving an absorbance value half that of neat samples.

Table 4.1

	Cell line	TN secretion (\pm SEM) (ng/10 ⁶ cells/ml/48h)	Number of times repeated
fibroblast	PEO9	519 (\pm 60)	2
	PEO12	1053 (\pm 213)	3
	PEO13	584 (\pm 266)	2
	PEO27	696 (\pm 269)	3
carcinoma	PEO1	<2	3
	PEO1CDDP	<2	3
	PEO4	<2	3
	SKOV-3	4.1 (\pm 2.7)	3
	59M	4.1 (\pm 3.9)	3

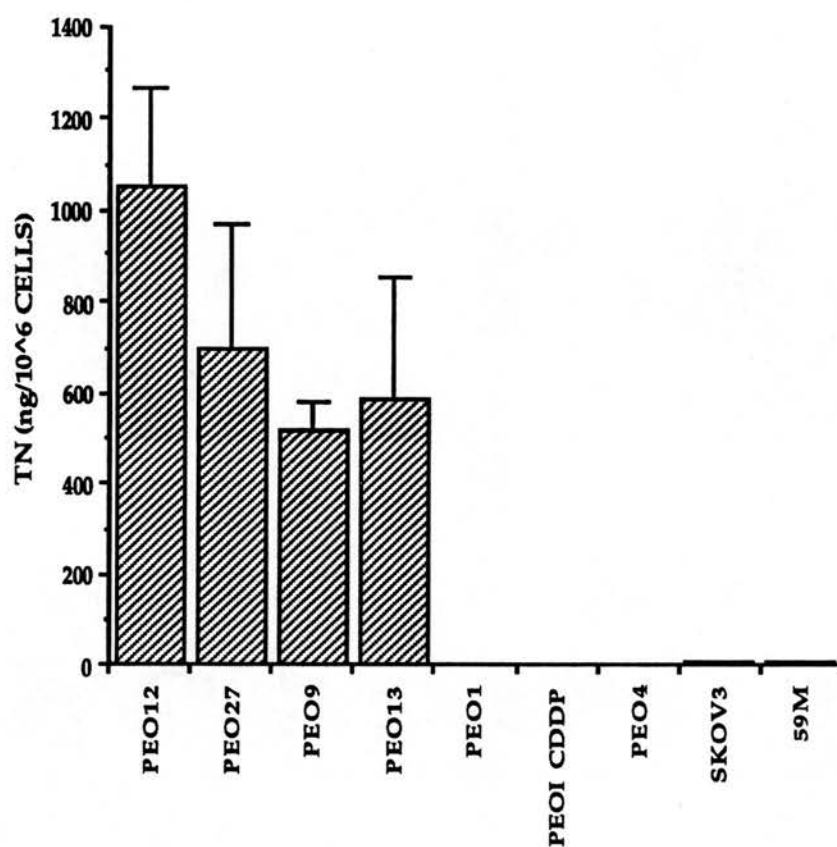


Figure 4.6 "Basal" level of TN secretion in ovarian fibroblast and carcinoma cell lines

The bars represent the mean level of secretion from a minimum of two experiments and the error bars show the standard error.

The fibroblast cell lines PEO9, PEO12, PEO13 and PEO27 secreted substantial amounts of TN into the media; at levels between 519 and 1053 ng/10⁶ cells/ml. The ovarian carcinoma cell lines SKOV-3 and 59M secreted approximately 4 ng/10⁶ cells/ml, while the remaining three carcinoma cell lines PEO1, PEO1^{CDDP} and PEO4 did not produce any measurable TN (the assay can accurately detect levels of TN as low as 2 ng per ml conditioned media).

It can be seen from these data that under unstimulated conditions fibroblasts secrete amounts of TN at least 100-fold greater than epithelial cells.

Conditioned media from the breast carcinoma cell lines, MDA-MB-231, T47D, MCF7 and ZR-75-1, were also assayed. Only the MDA-MB-231 breast cell line produced measurable levels of TN (an average of 8.7 (\pm 1.2) ng/10⁶ cells/ml). The remaining three cell lines secreted no detectable TN.

4.3 Tenascin RNA expression in cell lines.

The pattern of RNA transcripts for TN expressed by cell lines was examined using RT-PCR as described previously in section 3.2. The PCR gels produced were probed with the specific oligonucleotides also described in that section.

4.3.1 RNA preparation and validation of results

RNA was prepared from confluent flasks of cells. These cells were cultured in media containing 10% serum, as it proved difficult to grow cells to the required density, for efficient RNA extraction, in serum-free conditions. RNA was amplified from the ovarian fibroblast cell lines (PEO12 and PEO27) and ovarian carcinoma cell lines (PEO1, PEO1CDDP, PEO4, PEO14, PEA2, 2780AD, OVCAR4, SKOV-3 and 59M). Additional RNA samples were available from breast cell lines, (MDA-MB-231, T47D, ZR-75-1 and MCF-7). All RNA samples were prepared using tri-reagent (for liquid samples). RNA integrity was examined on RNA formaldehyde gels, an example of which can be seen in figure 4.7. All the samples in figure 4.7 are intact RNA. Two clear bands can be observed which correspond to the 18s and 28s subunits of ribosomal RNA. If an RNA sample had been degraded these distinct bands would not have been present and the RNA would appear as a smear down the gel.

Samples were reverse transcribed to cDNA and primers were used to detect the house keeping gene HPRT, as a positive control (figure 4.8). The HPRT product produced by these primers is 290 bp and can be observed as a single band. All samples illustrated in figure 4.8 contained this band and there was no contamination of any reagent with RNA as the negative control had no band.

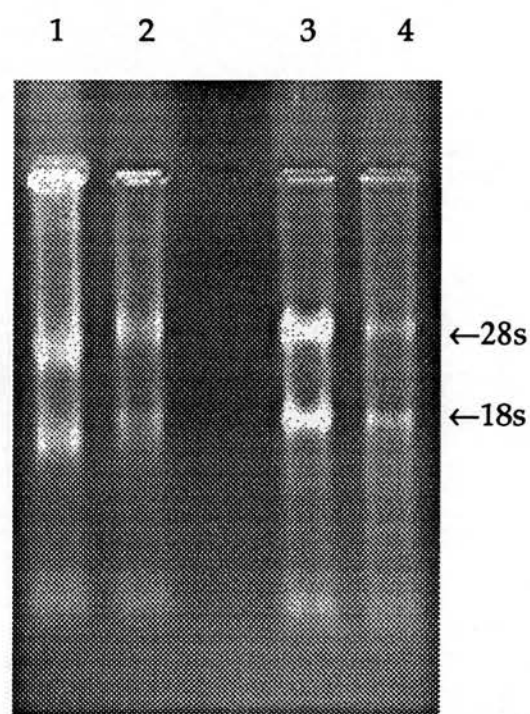


Figure 4.7 RNA formaldehyde gel.

1.1% formaldehyde-agarose gel, electrophoresed at 60 V for 2h. Samples 1 to 4 correspond to cell lines PEO1, PEO4 and tumours HOV 033, HOV 106.

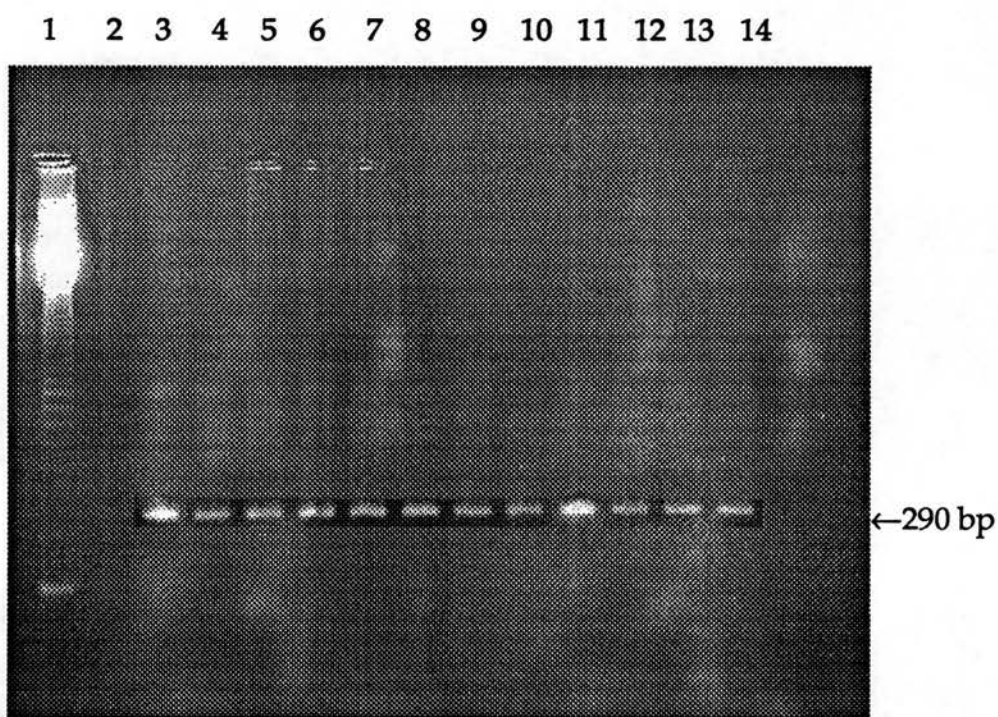


Figure 4.8 HPRT PCR products

1.4% agarose gel containing PCR products amplified for HPRT. Lane 1 contains a molecular weight ladder, lane 2 was a negative control containing no RNA. Lanes 3-9 correspond to RNA samples from ovarian cell lines (SKOV-3; 59M; PEO1; PEO4; PEO1 CDDP; PEO12; PEO27). Lane 10 contains the ovarian tumour HOV 171. Lane 11 contains a different RNA sample from PEO4 and lane 12 contains PEO12. Lanes 13 and 14 contain 2 samples of RNA from the breast carcinoma cell line MDA-MB-231.

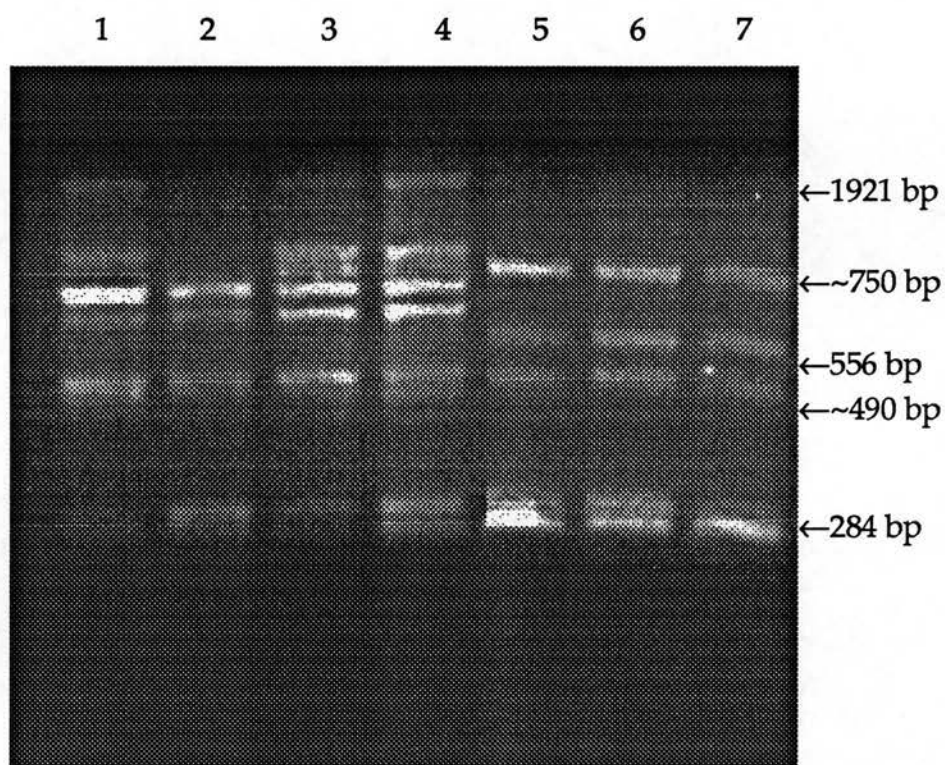


Figure 4.9 TN PCR products

Example of a typical gel from a PCR of TN. The lanes 1 to 5 contain the ovarian carcinoma cell lines SKOV-3, 59M, PEO1 and PEO4; lane 5 contains PCR products from the PEO12 fibroblast cell line; lanes 6 and 7 correspond to ovarian tumour samples HOV 091 and HOV 095.

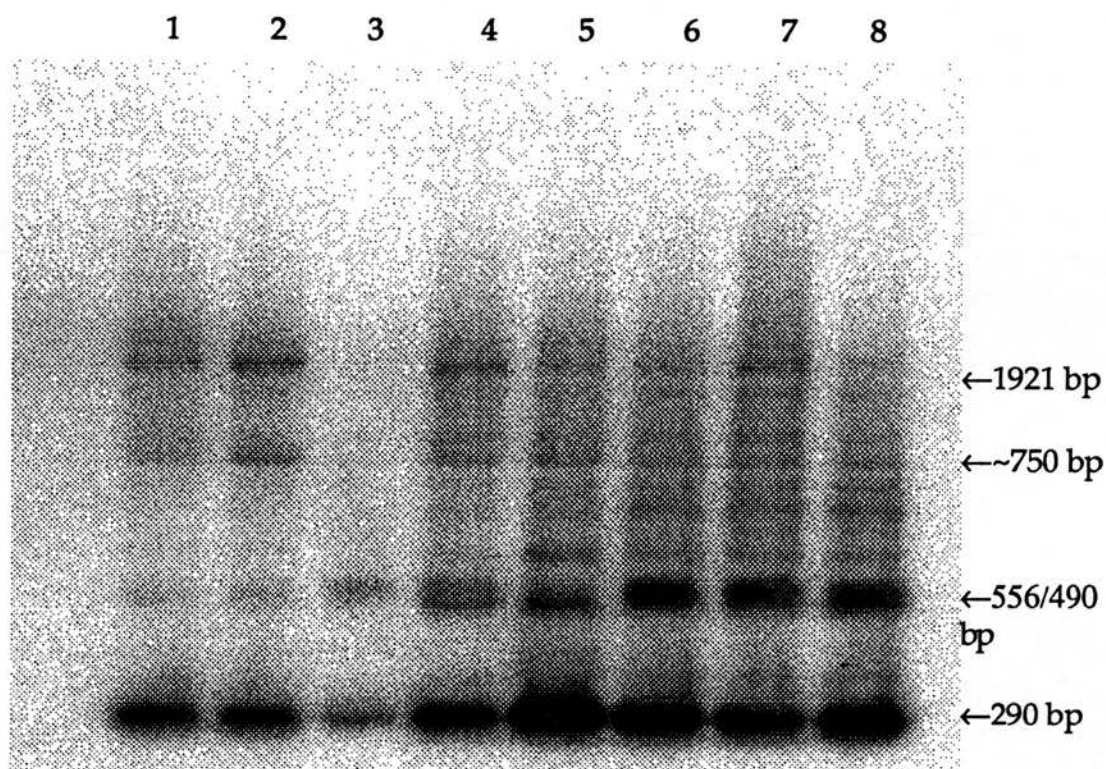


Figure 4.10 Probing of TN PCR products with oligonucleotide 5

The PCR products from a gel as observed in figure 4.7 were transferred to a nylon membrane and probed with oligonucleotide 5, which is capable of detecting all splice variants amplified by the PCR. All the bands observed on the gels were detected by this oligonucleotide, although some of the apparent doublets were difficult to distinguish. Lanes 1-3 contain malignant ovarian tumours (HOV 019, HOV 095, HOV 091), lane 4 contains the PEO12 fibroblast cell line and lanes 5-8 contain the ovarian carcinoma cell lines (PEO1, PEO4, 59M and SKOV-3)

4.3.2 Incidence of tenascin RNA in ovarian cell lines

When the cell line samples were amplified with the primers for TN a wide range of expression levels and patterns was observed. Figure 4.9 shows a typical gel in which the ovarian epithelial cell lines SKOV-3, 59M, PEO1 and PEO4 produced bands with molecular weights of approximately 284 bp, ~490 bp and a large number of bands of intermediate molecular weight (between 600 and 1921bp). The 556 bp form was notably absent in the ovarian carcinoma cell lines.

The PEO12 fibroblast sample produced fewer bands, although its pattern does appear to closely resemble that seen in the 2 ovarian tumour. The main bands in the fibroblast and tumour samples can be seen at 284 bp, ~490 bp, 556 bp, ~750 bp and 1921 bp. The ovarian fibroblasts and tumours did not produce the large number of bands of intermediate molecular weight which were observed in the carcinoma cell lines.

The amplified DNA was transferred to membranes and probed with the specific internal oligonucleotides described in section 3.2.1. Probing with oligonucleotide 5 (figure 4.10) verified the authenticity of the bands produced in the PCR reaction, as all the bands which were observed on the original gel hybridised with this probe.

A number of other ovarian carcinoma cell lines were also examined for TN PCR products. An additional ovarian fibroblast cell line was examined along with RNA from breast carcinoma cell lines. These data are summarised in table 4.2.

Table 4.2 Summary of TN RNA expression in cell lines

Cell type	Bands present (molecular weight in bp)				
	284	490	556	intermediate forms	1921
Ovarian carcinoma					
PEO1	+	+	-	multiple	+
PEO1CDDP	+	+	-	multiple	+
PEO4	+	+	-	multiple	+
PEO14	+	+	-	multiple	+
SKOV-3	+	+	-	multiple	+
59M	+	+	-	multiple	+
PEA2	+	+	-	multiple	+
2780AD	+	+	-	multiple	+
OVCAR4	+	+	-	multiple	+
Ovarian fibroblast					
PEO12	+	+	+	~750 only	+
PEO27	+	+	+	~750 only	+
Breast carcinoma					
MDA-MB-231	+	+	-	multiple	+
T47D	-	-	-	-	-
ZR-75-1	-	-	-	-	-
MCF7	-	-	-	-	-

From the data shown on the previous pages it can be seen that both the fibroblasts and ovarian carcinoma cell lines examined are capable of producing multiple forms of TN RNA. The ovarian fibroblasts produce a banding pattern strikingly similar to that observed in malignant ovarian tumours.

It is interesting to note that the ovarian carcinoma cell lines PEO1, PEO1CDDP and PEO4, which did not secrete any measurable TN into conditioned media, appeared to produce multiple molecular weight forms of TN RNA, i.e. the mRNA was not translated or it produced amounts of TN protein which were less than the sensitivity of the ELISA. The breast carcinoma cell lines T47D, ZR-75-1 and MCF7 were shown to produce no detectable TN RNA or protein. It is also possible that culturing the ovarian carcinoma cell lines in the presence of serum stimulated the production of TN RNA.

4.4 Modulation of tenascin secretion

The restricted distribution pattern of TN in human adults indicates that control of the expression of this molecule is highly regulated. Several growth factors, hormones and cytokines have been implicated in the modulation of TN expression; these factors may be acting in an autocrine, paracrine or even endocrine manner. This section describes experiments which examine paracrine influences and potential modulating factors of TN secretion in ovarian cell systems.

4.4.1 Co-culturing of cell lines

It was observed in the immunohistochemically stained ovarian tumour sections that the most intense TN staining was found in the stromal tissue adjacent to carcinoma cells (eg. figure 3.2). This pattern of staining would be consistent with the hypothesis that the secretion of TN by fibroblasts is stimulated by paracrine growth factors being secreted by the tumour cells.

In order to investigate whether such a paracrine loop exists in ovarian cell systems, ovarian fibroblasts (PEO12 and PEO9) were co-cultured with the PEO1 ovarian carcinoma cell line. The fibroblast and epithelial cell populations were kept physically separated using porous tissue culture inserts, as illustrated in figure 4.11 These inserts have a high density of pores to allow diffusion of soluble factors.

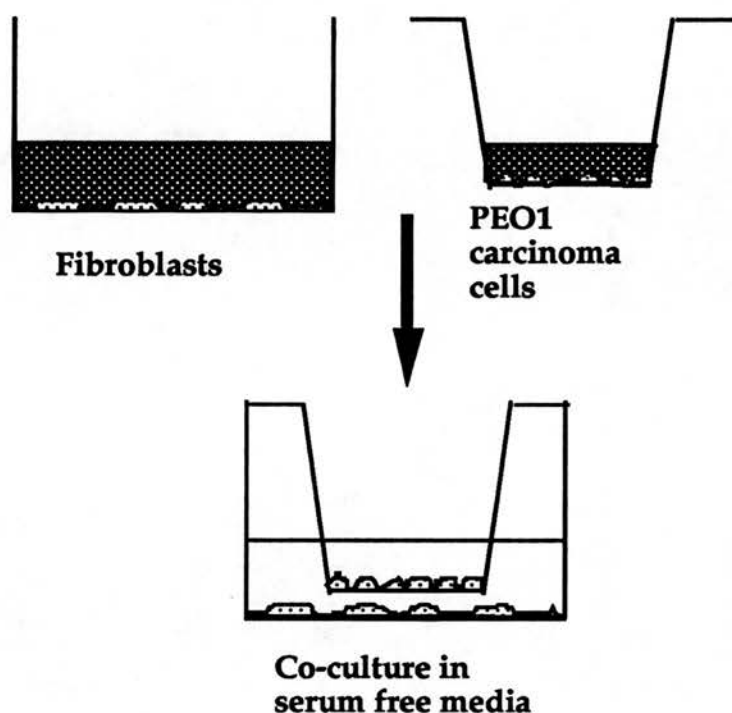


Figure 4.11 Schematic representation of the co-culturing method. The system was co-cultured for 48 h and the media was collected and assayed for TN content by ELISA.

The results of a typical experiment utilising both PEO12 and PEO9 fibroblasts are illustrated in figure 4.12.

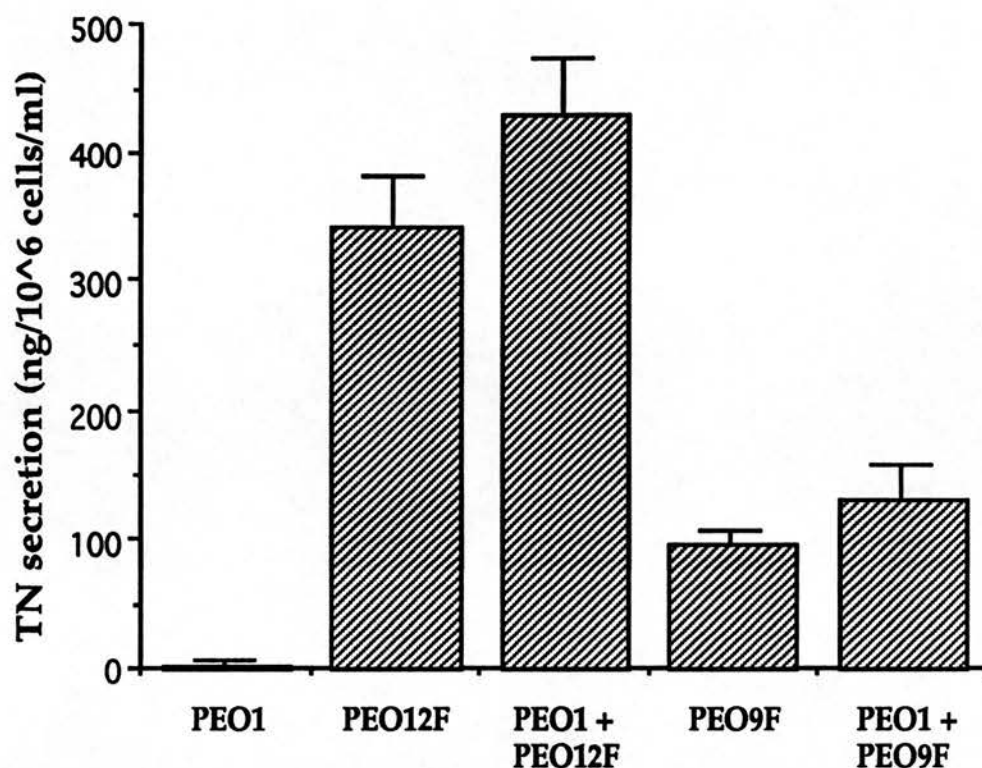


Figure 4.12 Levels of TN secretion in co-cultured populations of fibroblast and epithelial cells

Data are from a typical experiment. Each bar represents the mean value from quadruplicate wells and the error bars indicate the standard error.

The data in figure 4.12 demonstrates that PEO1 carcinoma cells secreted negligible amounts of TN, while both the PEO12 and PEO9 fibroblasts when cultured alone secreted considerable amounts of TN (342 and 98 ng/10⁶/ml respectively). When these fibroblasts were co-cultured with the PEO1 cells the level of TN secretion increased (to 432 ng/10⁶/ml for PEO12 and 131 ng/10⁶/ml for PEO9). The co-cultured cell populations secreted approximately 27% more TN than fibroblasts cultured alone. This experiment has been repeated and whilst the absolute values of TN secretion vary between experiments the degree of stimulation in the co-cultured populations remains the same.

In the mixed populations only the fibroblast cell number was taken into account, as the PEO1 cells were shown to secrete only background levels of TN. It is possible that the fibroblasts may have secreted factors which induced the PEO1 cells to secrete TN, however this is unlikely as further experiments (section 4.4.2 (ii)) indicate that PEO1 cells, even in the presence of TN stimulating factors, do not secrete levels of TN which would account for the differences observed in these co-culturing experiments.

In order to examine whether the increase in TN secretion in co-cultured populations was statistically significant, the amount of TN in the conditioned media containing fibroblasts alone was compared with the media from fibroblasts co-cultured with the epithelial cells using a paired statistical test. The data from experiments on 3 separate occasions was combined for analysis. The wells containing single and co-cultured populations were paired according to their position on the 24 well tray, with adjacent wells being analysed together.

The increase in TN secretion was observed in 11 of the 12 co-cultured populations. This was statistically significant with a p value of 0.0034, as tested by a Wilcoxon signed rank test.

4.4.2 Potential modulating factors of TN secretion.

A range of growth factors, hormones and cytokines was chosen on the basis of their modulations of TN secretion in other cell types and also on their effects on ovarian cell behaviour. The concentrations used for the initial screening had previously been established to be effective in ovarian cells.

Table 4.3 Concentrations used of potential modulating factors

Factor	Concentration	Reference
β -oestradiol (E2)	10^{-10} M	Langdon <i>et al.</i> , 1990
Human chorio gonadotrophin (HCG)	10 IU/ml	Langdon S.P. unpublished data
Follicle stimulating hormone (FSH)	0.1 IU/ml	Langdon S.P. unpublished data
Insulin-like growth factor-I (IGF-I)	10^{-8} M	Bartlett <i>et al.</i> , 1995
Insulin-like growth factor-II (IGF-II)	10^{-8} M	Bartlett <i>et al.</i> , 1995
Interferon γ (IFN)	10 IU/ml	Langdon S.P. unpublished data
Transforming growth factor- β_1 (TGF β_1)	2.4×10^{-9} M	Pearson <i>et al.</i> , 1988
Progesterone (PG)	10^{-10} M	Langdon S.P. unpublished data
Endothelin-1 (ET-1)	10^{-8} M	Moraitis <i>et al.</i> , in press

These factors did not cross react in the ELISA for TN.

(i) Modulation of fibroblast TN secretion.

The effects of adding the factors in table 4.2 to serum-free cultures of fibroblasts were investigated. The results of a typical experiment are shown in figure 4.13.

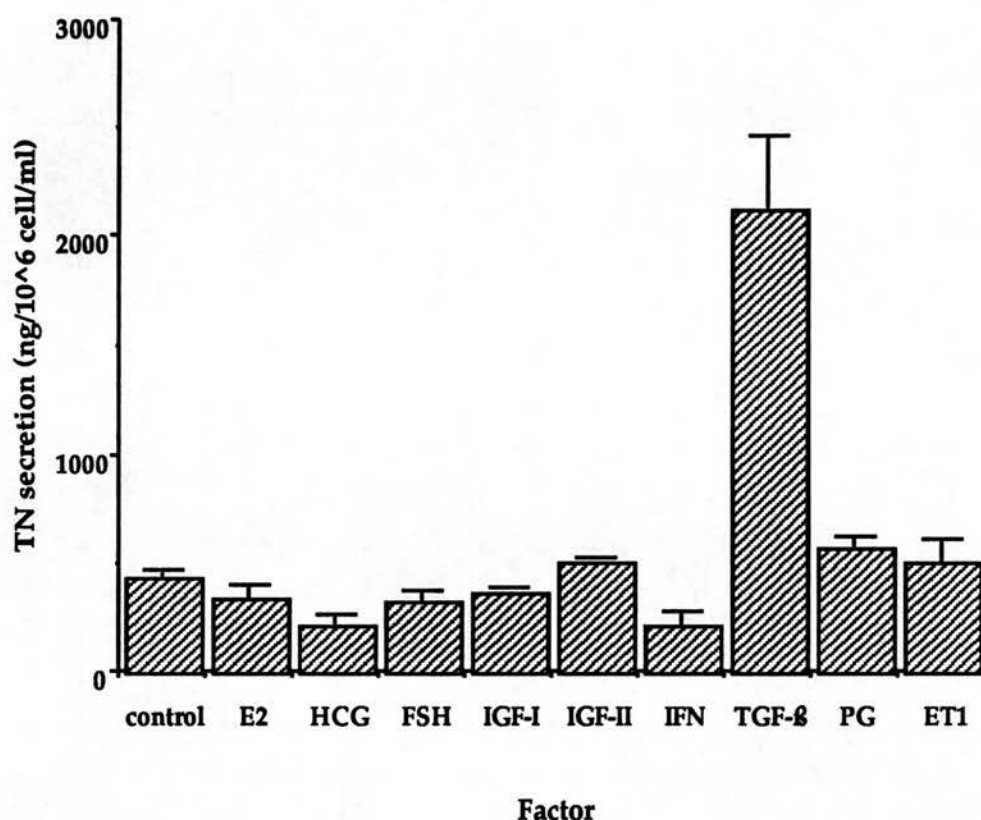


Figure 4.13 Modulation of TN secretion in the PEO12 fibroblast cell line

These data represent the absolute values of TN secretion from a single experiment. Each bar shows the mean value of triplicate wells and the error bars represent standard deviation.

In this experiment stimulation of TN synthesis was observed upon addition of IGF-II, TGF-β₁ and progesterone. Addition of HCG, FSH, IFN and IGF-I produced inhibition of TN secretion. Statistical analysis using Dunnett multiple comparisons test demonstrated that TGF-β₁ was the only factor to produce a significant effect with a p value less than 0.01. Levels of TN secretion after addition of oestrogen and endothelin were not different to control levels. This experiment was repeated and the data from those factors which were found to produce a consistent effect are

illustrated in figure 4.14.

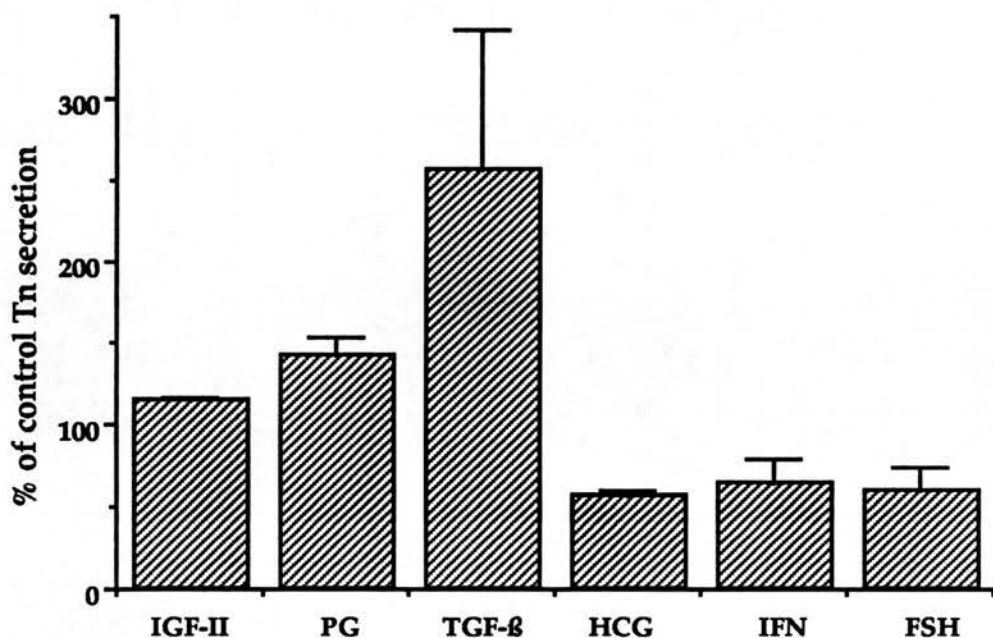


Figure 4.14 Percentage modulation of TN secretion in the PEO12 fibroblast cell line.

These data represent the mean values from a minimum of two experiments. TN secretion is expressed as the percentage of the control level of secretion with the control level being equal to 100%. For an individual experiment each factor was assayed either in quadruplicate or triplicate. The error bars represent the standard error. All these factors produced an effect significantly different from control levels

TN secretion in the PEO12 ovarian fibroblast cell line was stimulated by IGF-II (115%), progesterone (142%) and TGF- β_1 (257%). TGF- β_1 produced the largest induction of TN with a mean 2.5 fold increase. While the effects of IGF-II and progesterone were relatively small they consistently stimulated TN secretion above control levels. Conversely, TN secretion was inhibited by the gonadotrophins, HCG (56%) and FSH (59%) and by interferon (65%). HCG produced the greatest effect decreasing TN secretion to approximately 50% of control levels.

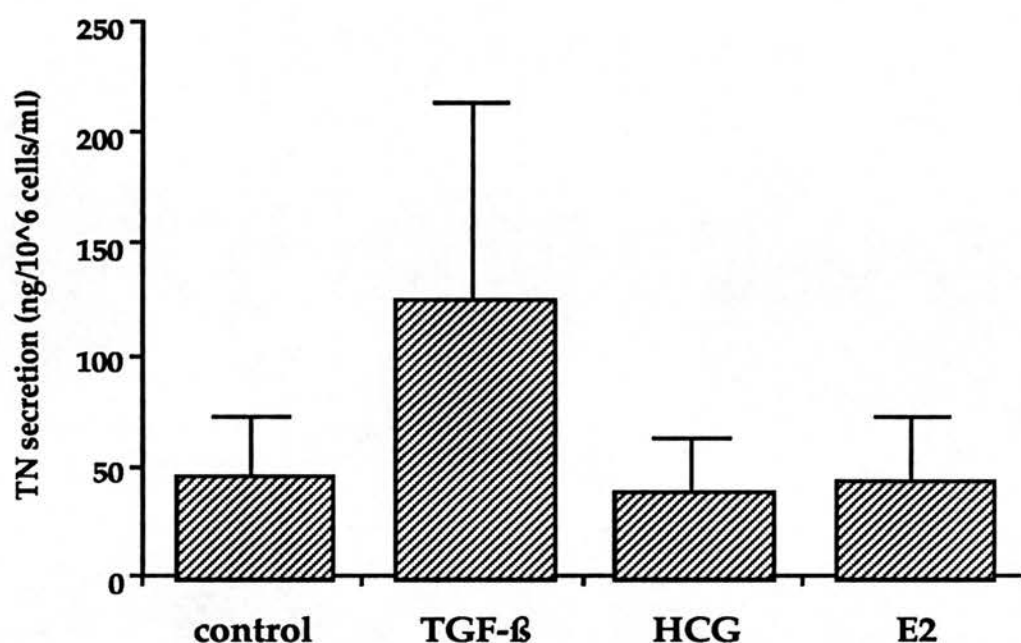


Figure 4.15 Modulation of TN secretion in the PEO27 fibroblast cell line

This graph represents the absolute values of TN expression in the PEO27 cell line, each bar shows the mean level of secretion from duplicate wells and the error bars show the standard deviation.

Similar results, to those observed in the PEO12 cell line, were obtained with the PEO27 cell line, although it produced less TN under basal conditions. There was a 2.8 fold induction in TN secretion upon addition of TGF- β_1 (although this was not significantly different from the control in this experiment).

(ii) Modulation of carcinoma cell TN secretion

Although most of the carcinoma cells tested failed to secrete measurable levels of TN in serum-free conditions, the range of factors was also tested against the PEO1 ovarian carcinoma cell line and the MDA-MB-231 breast carcinoma cell line, which does secrete measurable levels of TN.

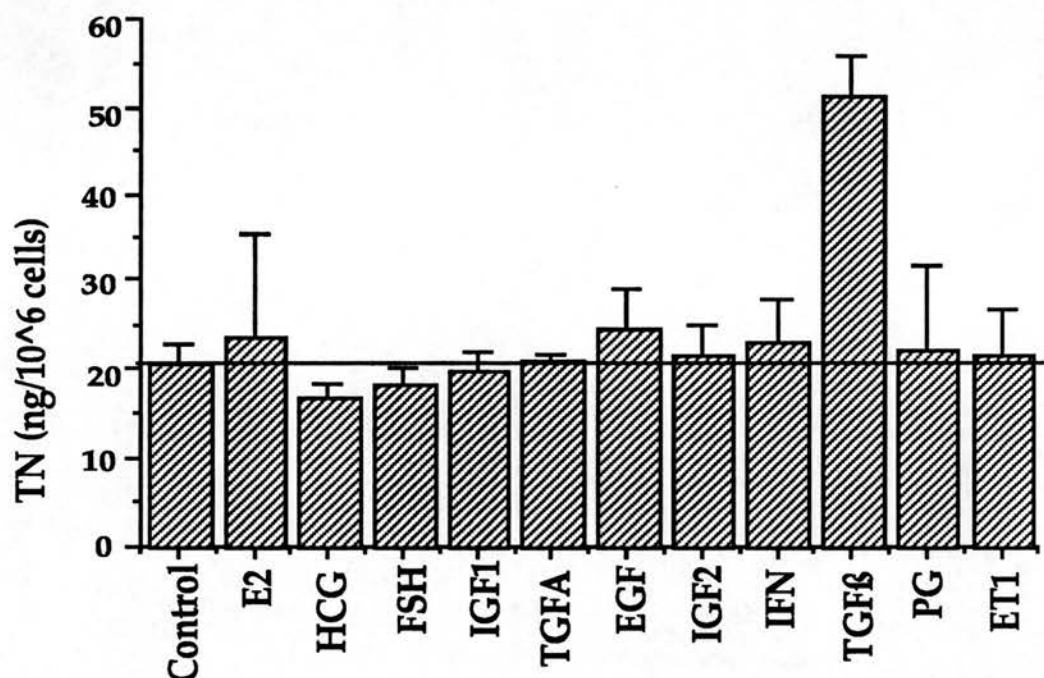


Figure 4.16 Modulation of TN secretion in the breast carcinoma cell line MDA-MB-231

This graph illustrates absolute values of TN secretion by the MDA-Mb-231 breast carcinoma cell line. Each bar represents the mean level of secretion from triplicate wells and the error bars represent the standard deviation. The line indicates the control level of secretion for reference.

The only factor of those tested to alter the level of secretion, in MDA-MB-231 cells, was TGF- β_1 which increased the levels of secreted TN by a factor of 2.5 ($p = < 0.01$ by Dunnetts multiple comparison test).

The factors TGF β_1 , EGF, TGF α and IGF-II were also tested against the PEO1 ovarian carcinoma cell line, which secretes no measurable TN in serum-free conditions. None of these factors increased the level of TN to a detectable level. This does not definitively demonstrate that PEO1 cells never produce TN, although they do not appear to be capable of secreting levels which are comparable with those produced by ovarian fibroblasts, or the breast cell line MDA-MB-231.

4.4.3 Modulation of TN secretion by TGF- β_1

In PEO12 fibroblasts, PEO27 fibroblasts and MDA-MB-231 breast cancer cells, TGF- β_1 produced the largest stimulation of TN secretion of all the factors tested. To investigate whether TGF- β_1 exerted its effects in a dose dependent manner, the factor was added in a range of concentrations as used by Pearson *et al.* (1988). 60 ng/ml is equivalent to the 2.4×10^{-9} M concentration used in the initial screening.

Figure 4.17 illustrates the effects of TGF- β_1 on the level of TN secretion by PEO12 fibroblasts

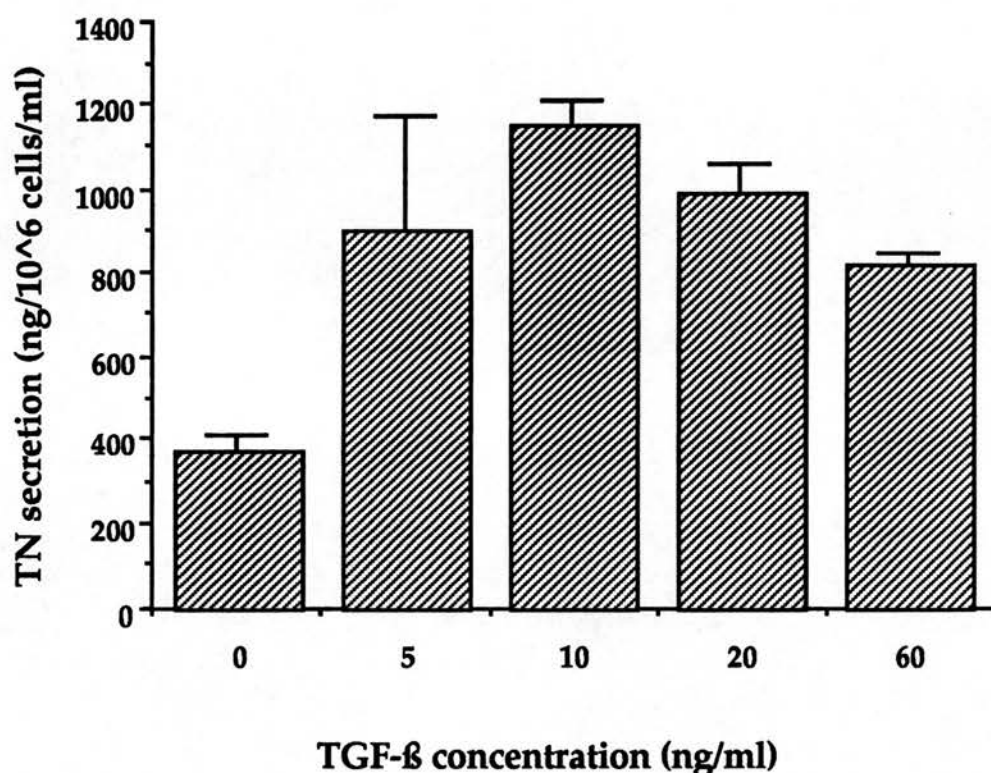


Figure 4.17 Modulation of TN secretion in PEO12 fibroblasts by TGF- β_1

This graph represents mean values of TN secretion from quadruplicate wells. The error bars represent standard deviation.

All concentrations of TGF- β_1 produced a significant stimulatory response (p values less than 0.01 by Dunnett multiple comparison test) The maximum stimulation was observed upon addition of 10 ng/ml TGF- β_1

The experiment was repeated in a modified fashion to include a 10 fold dilution of TGF- β_1 . The TGF- β_1 is dissolved in 4mM HCl containing BSA (1 mg/ml), therefore this experiment contained an additional control of serum free media containing BSA and HCl to the same concentrations as were found in the TGF- β samples

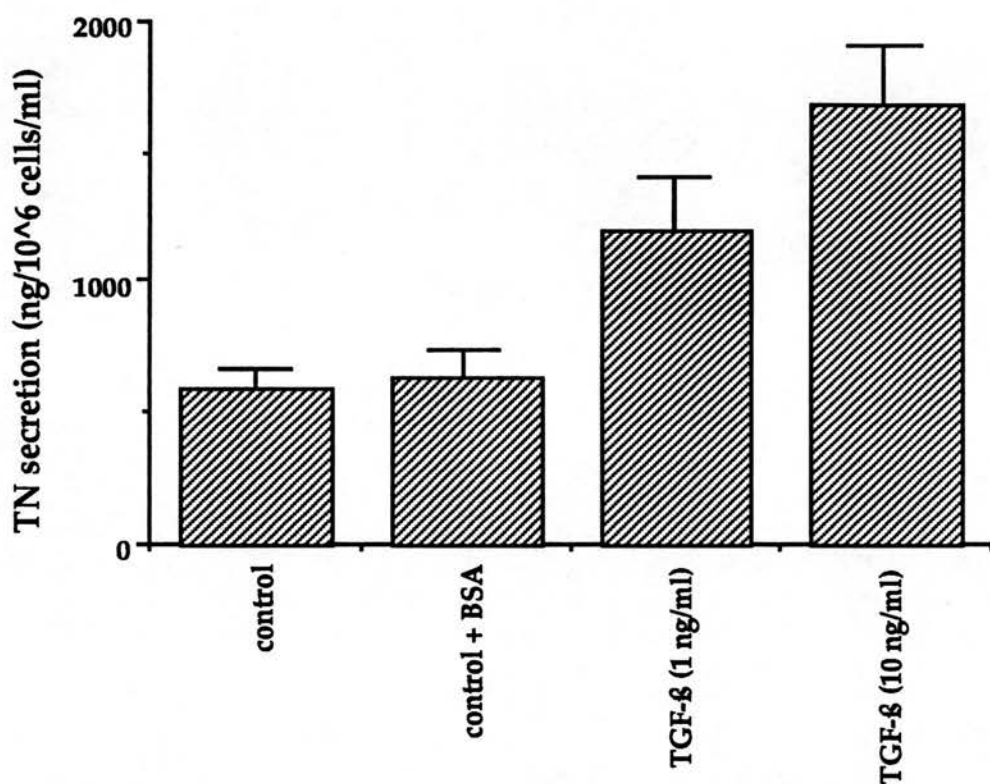


Figure 4.18 Modulation of TN secretion in PEO12 fibroblasts by TGF- β_1

This graph illustrates the mean values of TN secretion from quadruplicate wells, the error bars represent standard deviation.

These data demonstrate that TGF- β_1 stimulates the secretion of TN in a dose dependent manner. The effect is specific to the TGF- β , as the acidified BSA solution the factor is dissolved in has no effect on the level of TN secretion

Chapter 5.

Functional roles of Tenascin

Chapter 5.

Functional roles of Tenascin

The previous two chapters have demonstrated that TN expression is increased in ovarian carcinomas and that ovarian fibroblasts are capable of secreting TN into their surroundings. The secretion of TN by fibroblasts can be increased by growth factors such as TGF- β_1 . This chapter describes experiments which were conducted to assess whether TN is functional in ovarian cell systems. Previous studies of other carcinoma systems have indicated that TN may facilitate invasion or stimulate cell proliferation and these properties were investigated.

5.1 Attachment of ovarian carcinoma cell lines to ECM proteins

The matrix of proteins surrounding cells not only provide structural support, but it is now appreciated that changes in this matrix environment can lead to altered cell morphology and behaviour, such as increased migration and proliferation. For the matrix proteins to exert these effects cells must physically attach to the proteins. The experiments in this section examined the attachment of ovarian carcinoma cells to TN, both quantitatively and qualitatively. The effects of TN were compared with those of fibronectin, with which TN shares some homology, and collagen IV, which is a major component of most matrices.

5.1.1 Adhesion of cells to ECM proteins

These experiments investigated the binding of cells to ECM proteins, using a quantitative assay in which aliquots of radiolabelled cells were incubated in wells containing the ECM proteins; additional aliquots of cells were placed directly into counting tubes to provide the input value. The percentage adhesion was determined by comparing the residual radioactivity in the wells with this input value.

96 well trays were coated with TN by incubation overnight at 4°C; the ECM proteins fibronectin (FN) and collagen IV (CIV) were also coated

onto the trays for comparison. Bovine serum albumin (BSA) (1%) was used as a control to examine any non-specific protein binding; the binding to BSA was always less than 10% of the input value, this nonspecific binding was subtracted from all the values before the percentage adhesion was calculated.

(i) Determination of saturating protein levels.

The majority of ECM proteins produce saturating levels of cell binding at 10 $\mu\text{g/ml}$ (JF Marshall, personal communication), however there are no data available for the ovarian cell lines and relatively little data on the binding properties of TN. Serial dilutions of the proteins were used to determine the saturating concentrations for these proteins with the ovarian carcinoma cell lines. The cell lines to be investigated were radio-labelled with chromium and incubated in the trays for 2h to allow attachment to the ECM proteins, before washing and counting.

The results from this experiment using the SKOV-3 ovarian carcinoma cell line are illustrated in figure 5.1.

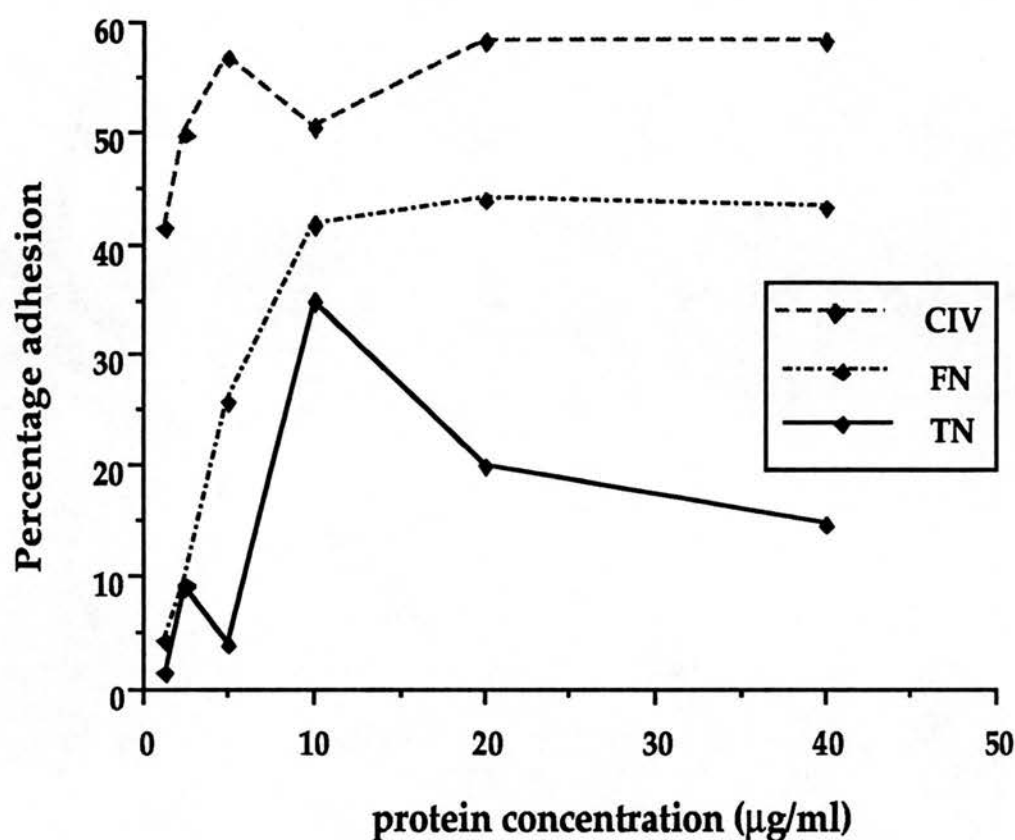


Figure 5.1 Attachment of SKOV-3 cells to different concentrations of ECM proteins

These data represent the levels of binding to collagen IV (CIV), fibronectin (FN) and tenascin (TN). The points on this graph represent the mean data from quadruplicate wells, error bars are not illustrated however the percentage standard deviation was always less than 10 %

It was observed in the above experiment that as the concentration of fibronectin and collagen IV increased adhesion on fibronectin increased up to a plateau where 42% of the cells had adhered; collagen IV showed a similar effect with a maximum level of 58% adhesion within the 2 hour incubation period. The levels of fibronectin and collagen IV appeared to reach saturating levels at 10 and 20 μg/ml respectively. Binding to TN increased to a peak at 10 μg/ml of protein and then the level of adhesion

decreased. For further experiments the ECM proteins were used at a concentration of 10 $\mu\text{g/ml}$; as binding is optimal or near optimal in most instances.

(ii) Comparison of attachment of different cell lines to ECM proteins

The ligand preferences of the ovarian carcinoma cell lines were investigated by plating the cells onto TN, fibronectin and collagen IV, all at 10 $\mu\text{g/ml}$.

The results from a typical experiment are shown in figure 5.2.

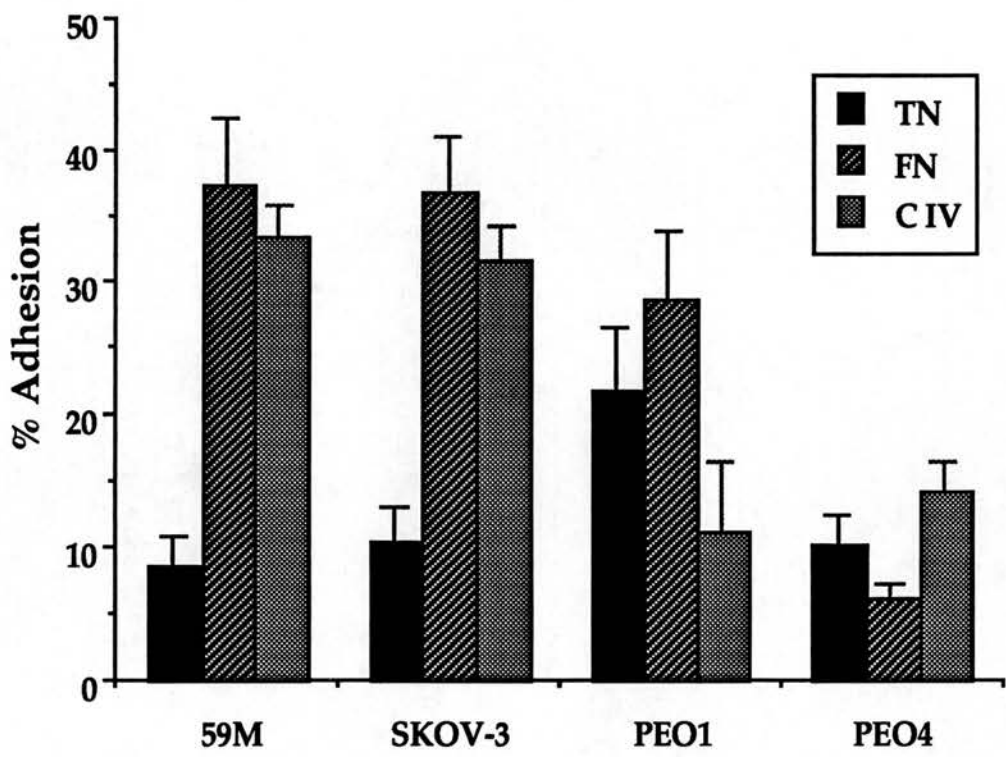


Figure 5.2 Adhesion of ovarian carcinoma cells to ECM proteins

The bars represent the mean value from quadruplicate wells and the error bars show the percentage standard deviation. The ECM proteins were used at a concentration of 10 $\mu\text{g/ml}$. (TN =tenascin; FN =fibronectin; C IV =collagen IV).

The SKOV-3 and 59M cell lines show a similar level of adhesion; in the wells containing fibronectin and collagen IV around 40% of the cells attached. In the wells containing TN, attachment was considerably less, approximately 10% of the input value. The level of attachment of these cells to TN was significantly different to that observed on fibronectin and collagen IV ($p < 0.001$ by Tukey-Kramer multiple comparison test).

The PEO1 cell line showed a different preference for the ECM proteins. The greatest level of adhesion (29%) was observed on fibronectin with only a slightly reduced level of binding to TN (22%); these levels of adhesion were not significantly different from each other. The poorest substrate for adherence of PEO1 cells was collagen IV which demonstrated only 11% adhesion; a level of binding significantly different to both TN and fibronectin ($p < 0.05$ and $p < 0.001$ respectively by Tukey-Kramer multiple comparison test).

PEO4 cells demonstrated the lowest levels of adhesion overall, with maximum levels of 14% seen on collagen IV. They showed the least preference for fibronectin (6%) and slightly more binding to TN (10%). The levels of binding to TN and fibronectin were not significantly different to each other.

This experiment has subsequently been repeated and while the absolute values of adhesion differ between experiments, relative amounts of binding to the different proteins remained the same. All values were corrected for non-specific protein binding by subtracting the level of adhesion to the wells containing BSA (the level of attachment to BSA was always less than 10% of the input value and was comparable to the level of adhesion observed on uncoated plastic). Therefore, it can be seen from these data that TN does promote the attachment of ovarian carcinoma cells, although different cell lines have varying affinities for the ECM proteins studied.

5.1.2 Morphology of cells on ECM proteins

The morphology of the cell lines was examined qualitatively by noting

the appearance of the cells attached to TN, fibronectin and collagen IV, by phase contrast light microscopy. Cells which attached well were less refractile than those which were weakly attached. BSA was again used to provide a control for non-specific adhesion of cells to protein; very small numbers of cells attached to the BSA, comparable to those attaching to uncoated well surfaces.

Figures 5.3 to 5.6 show the morphology of the four cell lines investigated

In routine culture PEO1 cells do not show a large degree of spreading and appear rounded. However, in this experiment (figure 5.3a) it was observed that there were differences in morphology of the cells plated on TN and fibronectin; the cells on TN appear more refractile, implying that they have not spread out on the substrate. Fewer cells appeared to have attached to the collagen IV and very few cells were attached to the BSA coated surface.

As was observed in the adhesion assay, the PEO4 cells showed the lowest levels of attachment to all proteins (figure 5.4). The cells that attached on TN appeared to be rounded and refractile with little obvious spreading when compared to those on fibronectin and collagen IV. The lowest level of attachment was on BSA and collagen appeared to provide the best substrate for attachment.

The SKOV-3 cells attached and spread in large numbers on all the proteins with the exception of BSA (figure 5.5). It was observed that cells on TN had a different morphology to those on fibronectin and collagen IV. The morphology on TN was best described as "spindly". This different morphology may mean that the cells were not so firmly attached and could explain why the level of attachment measured in the adhesion assay was so much lower, since these cells may have been dislodged during the washing process.

59 M cells did not attach to BSA coated wells however, they attached and spread on the ECM proteins (figure 5.6). No obvious differences in morphology were observed between the 3 proteins.

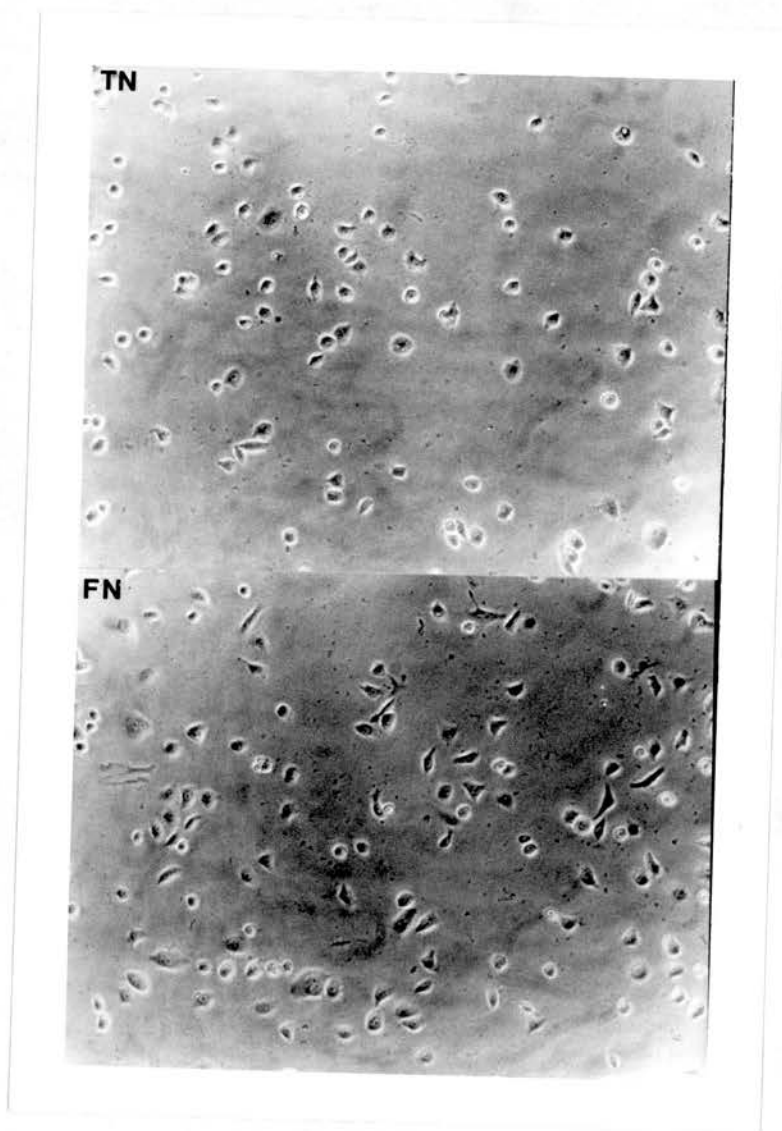


Figure 5.3 a Morphology of PEO1 cells on TN and fibronectin

This figure illustrates the morphology of PEO1 cells on TN and fibronectin (FN) as observed under phase contrast microscopy. (Magnification x100)

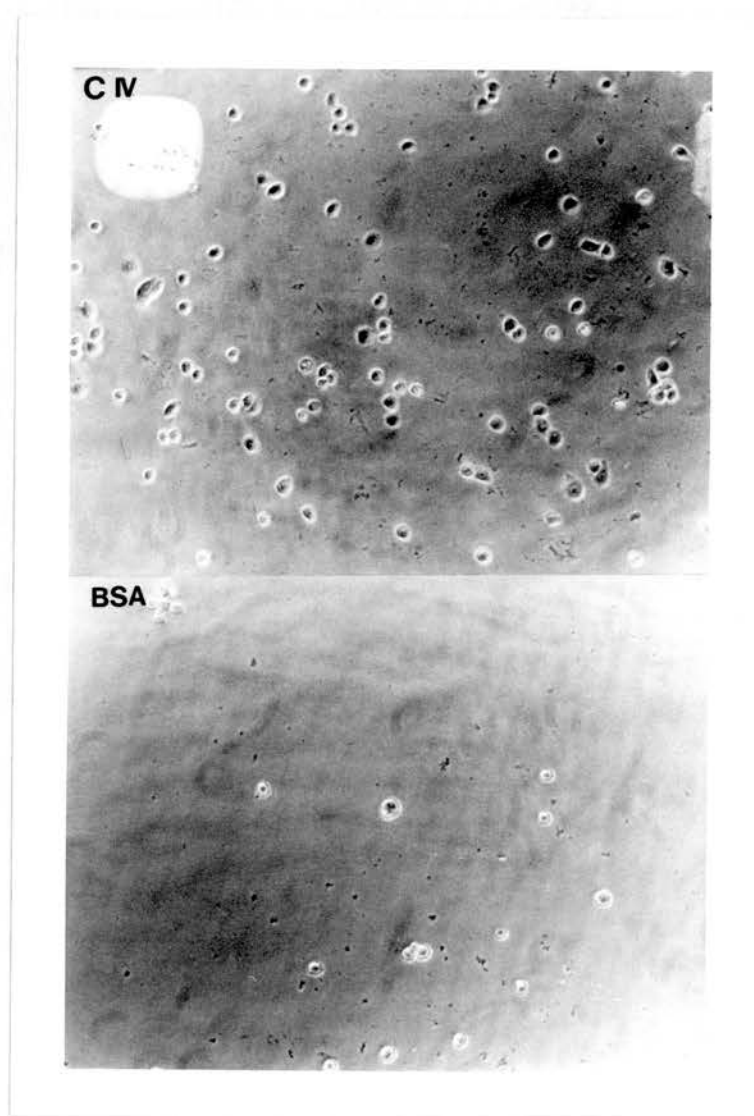


Figure 5.3 b Morphology of PEO1 cells on collagen IV and BSA

This figure illustrates the morphology of PEO1 cells on collagen IV (CIV) and BSA as observed under phase contrast microscopy. (Magnification x100)

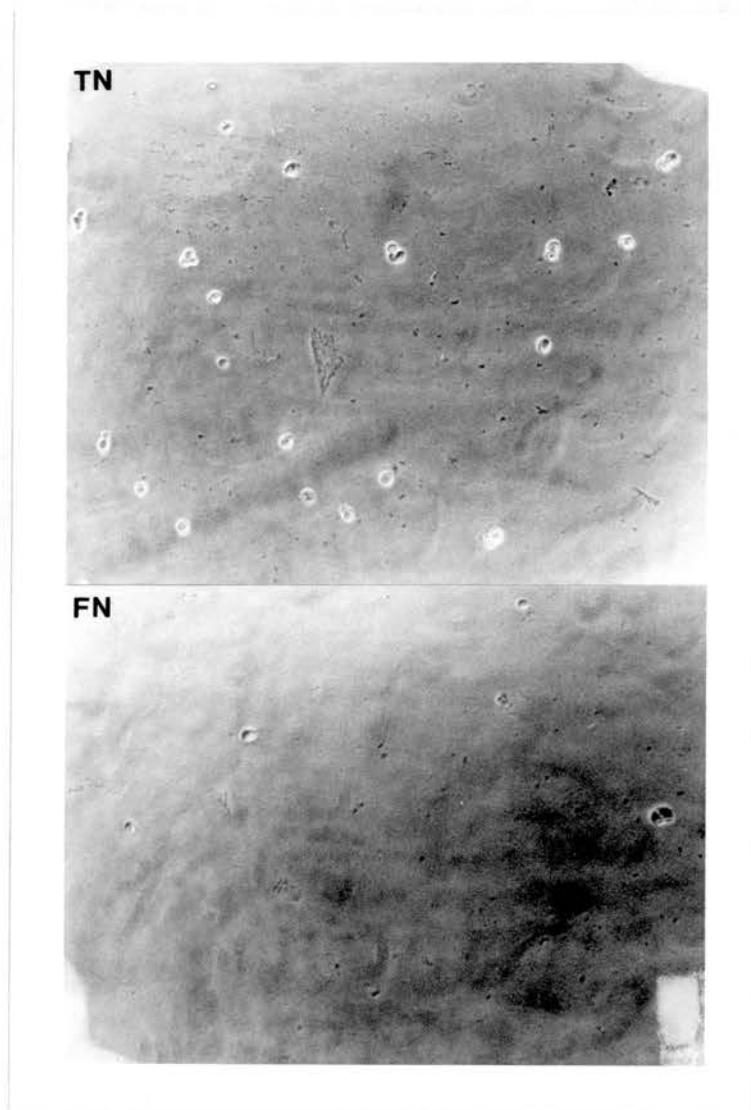


Figure 5.4 Morphology of PEO4 cells on TN and fibronectin

This figure illustrates the morphology of PEO4 cells on TN and fibronectin (FN) as observed under phase contrast microscopy. (Magnification x100)

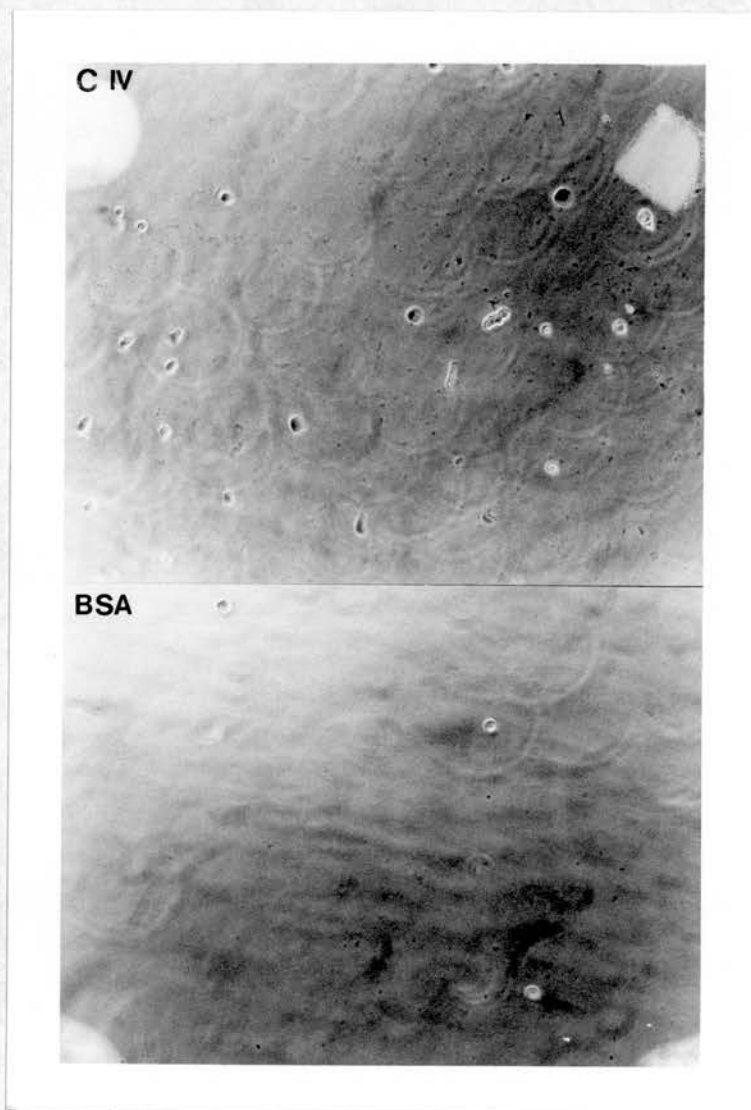


Figure 5.3 b Morphology of PEO4 cells on collagen IV and BSA

This figure illustrates the morphology of PEO4 cells on collagen IV (CIV) and BSA as observed under phase contrast microscopy. (Magnification x100)

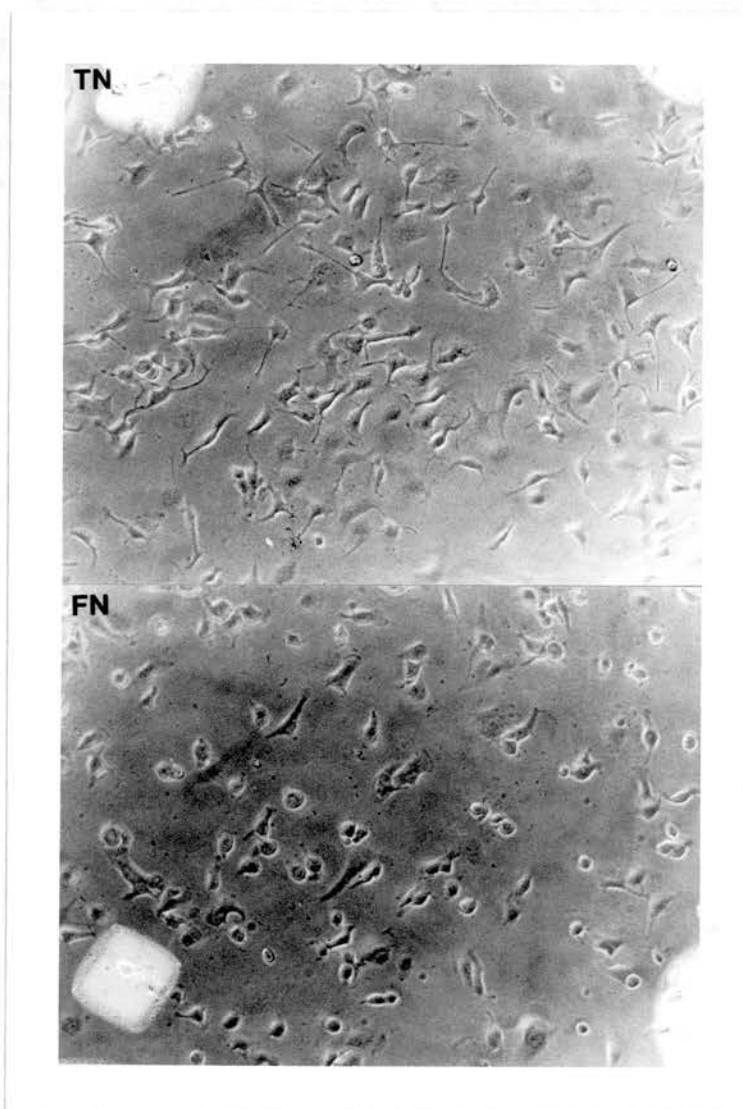


Figure 5.4 Morphology of SKOV-3 cells on TN and fibronectin

This figure illustrates the morphology of SKOV-3 cells on TN and fibronectin (FN) as observed under phase contrast microscopy. (Magnification x100)

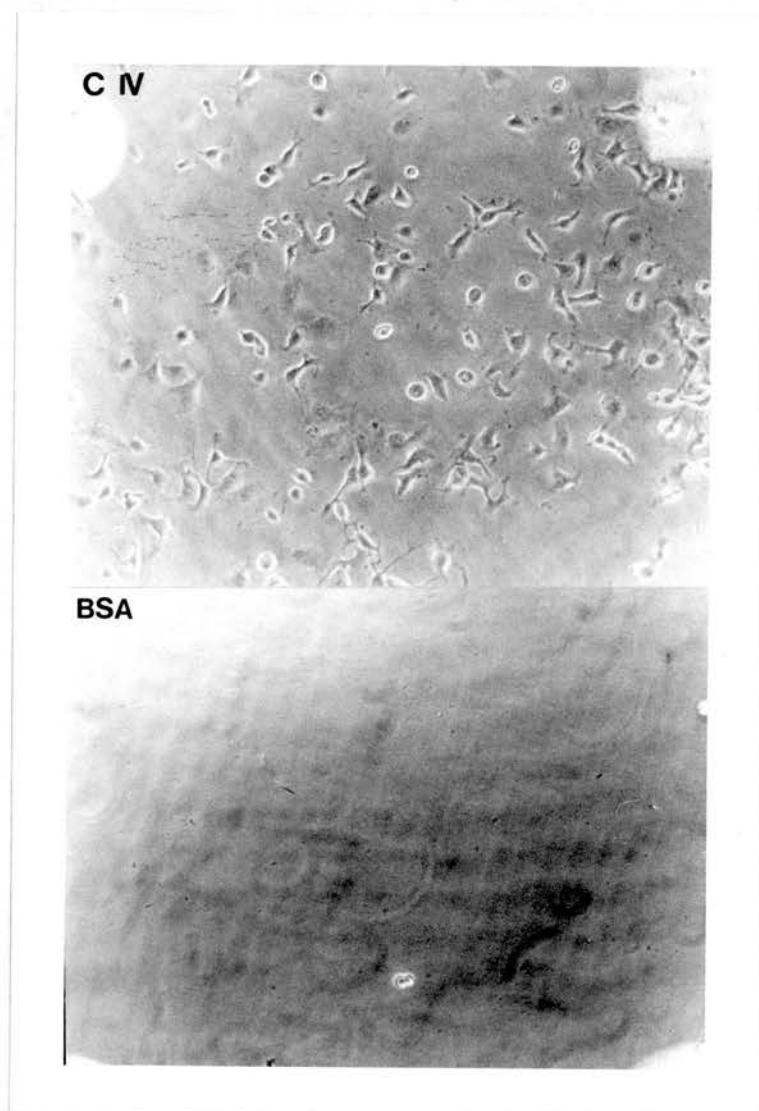


Figure 5.3 b Morphology of SKOV-3 cells on collagen IV and BSA

This figure illustrates the morphology of SKOV-3 cells on collagen IV (CIV) and BSA as observed under phase contrast microscopy. (Magnification x100)

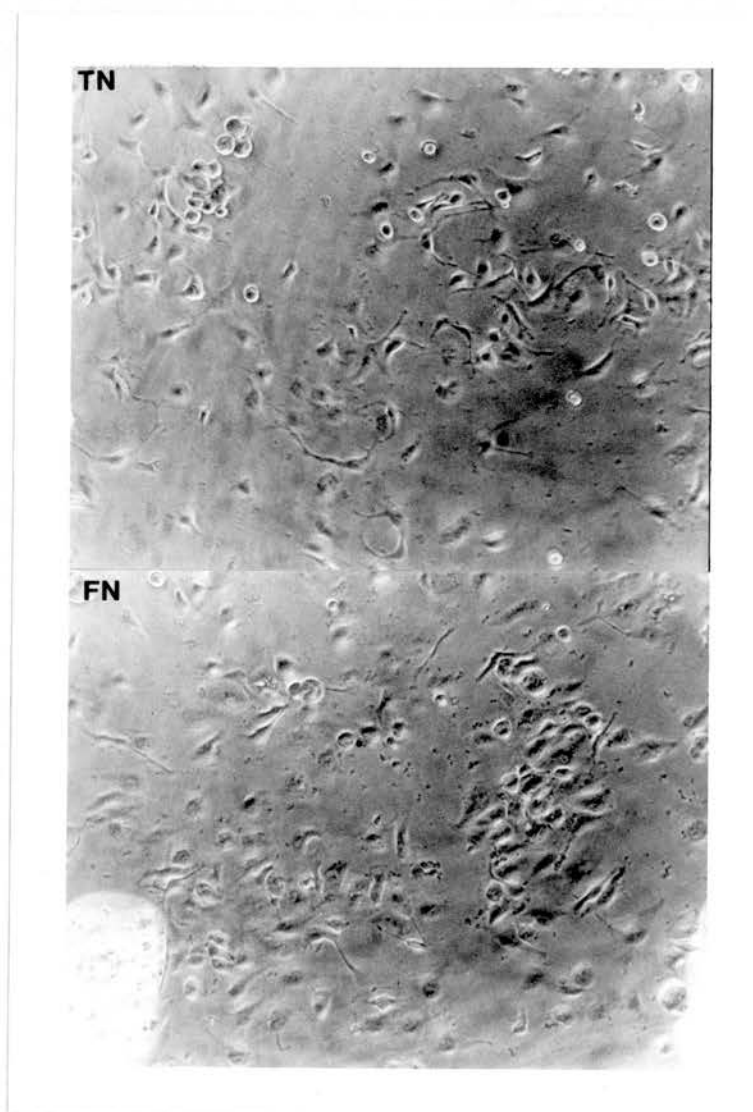


Figure 5.4 Morphology of 59M cells on TN and fibronectin

This figure illustrates the morphology of 59M cells on TN and fibronectin (FN) as observed under phase contrast microscopy. (Magnification x100)

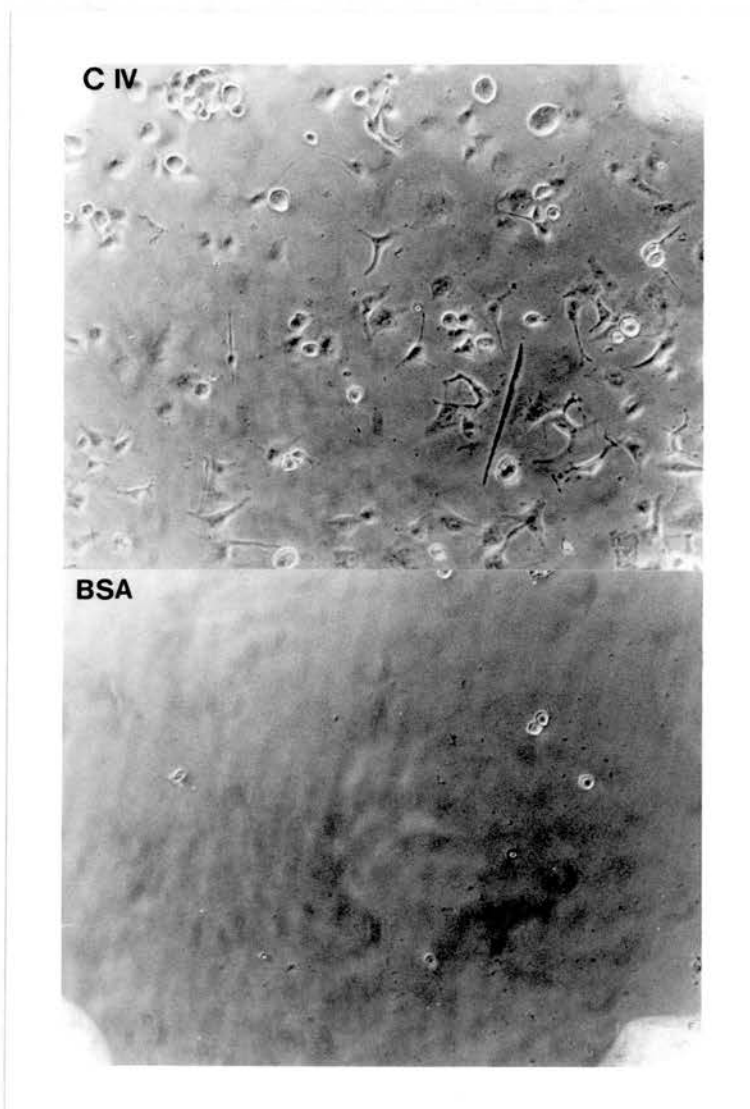


Figure 5.3 b Morphology of 59M cells on collagen IV and BSA

This figure illustrates the morphology of 59M cells on collagen IV (CIV) and BSA as observed under phase contrast microscopy. (Magnification x100)

5.2 Migration of ovarian carcinoma cells on ECM proteins

The haptotactic motility of cells on TN, fibronectin and BSA was measured by examining the migration of cells through porous transwell inserts. The lower surface of the inserts were coated with each respective protein (10 $\mu\text{g/ml}$). The cells were added to the upper surface of the membrane in serum-free medium. The lower compartment also contained serum-free medium with no chemoattractants, as the assay measured directional migration on substrate bound ECM proteins. The cells were incubated at 37°C for 48 h, to ensure that all cells had an opportunity to adhere to the transwell surface.

The relative proportions of cells on the under side of the transwells were determined, in initial experiments, by counting 3 random high powered microscope fields, as illustrated in figure 5.7a. This method was problematic and time consuming, as the high density of pores in the membrane made identifying cells difficult. This was overcome by staining the cells on the underside of the membrane with crystal violet. This stain could then be solubilised and measured spectroscopically (figure 5.7b).

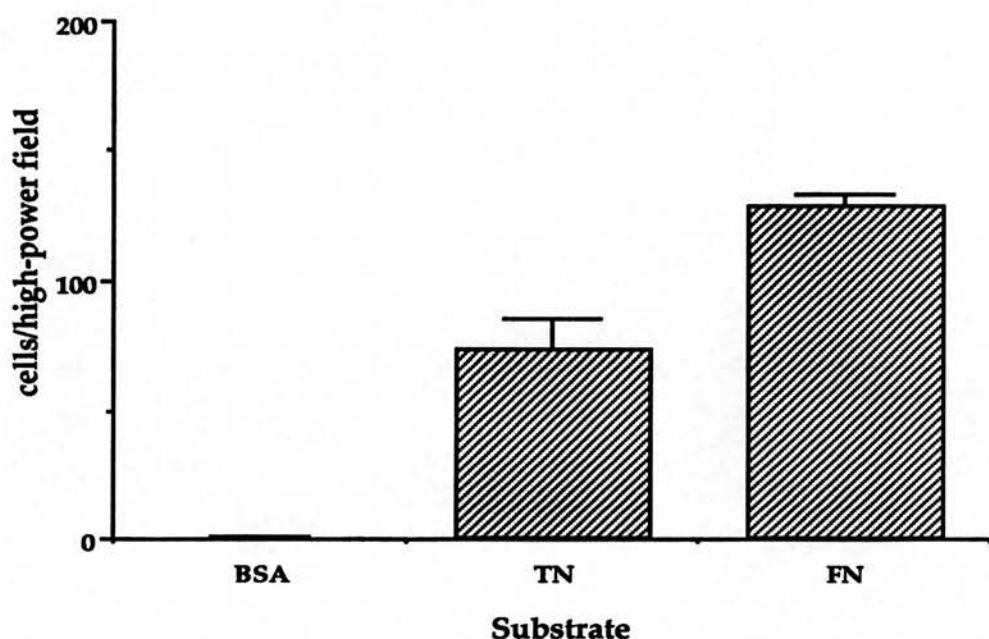


Figure 5.7a Migration of SKOV-3 cells, by counting random fields

These data represent mean number of cells/high powered microscope field from triplicate wells, for each well the mean number of cells was determined by counting 3 random fields. The error bars represent standard deviation.

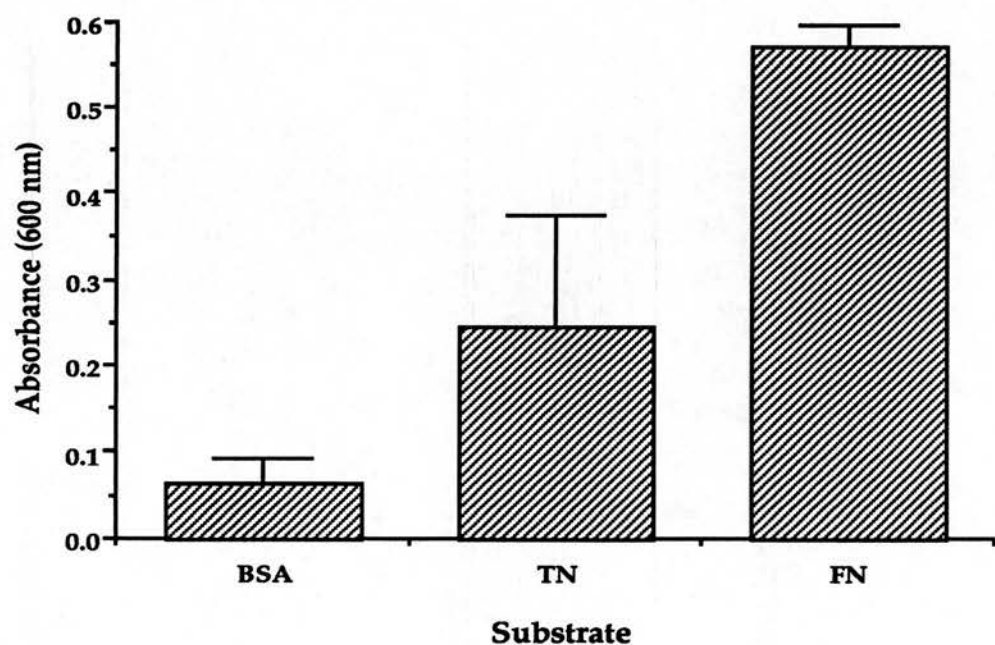


Figure 5.7b Migration of SKOV-3 cells measured by crystal violet staining

This figure shows the mean data from triplicate wells, with error bars representing standard deviation.

The data in figure 5.7 a demonstrates that very few cell migrated through the transwell coated with BSA (0.43 cells/field), while relatively large numbers of cells were found on the underside of the transwells coated with TN and fibronectin (74 cells/field and 129 cells/field respectively) Figure 5.7 b confirms these findings with a low level of crystal violet staining on BSA treated filters. Higher absorbance values, corresponding to larger numbers of stained cells, were observed in the transwells coated with TN and fibronectin. Thus both methods for examining the number of cells on the underside of the membrane produced similar results, with BSA showing very low levels of migration, TN and fibronectin promoting migration. Significantly more cells apparently migrated on fibronectin than TN ($p = <0.01$ by Student-Newman-Keuls multiple comparison test).

Both these methods are commonly used to assess migration, although they do not take into account the number of cells remaining in the upper compartment of the transwell. A more revealing method for assessing migration therefore is to count cells in both the upper and lower compartments and express migration as the percentage of cells which have passed through the membrane. A typical result from such an experiment is shown in figure 5.8

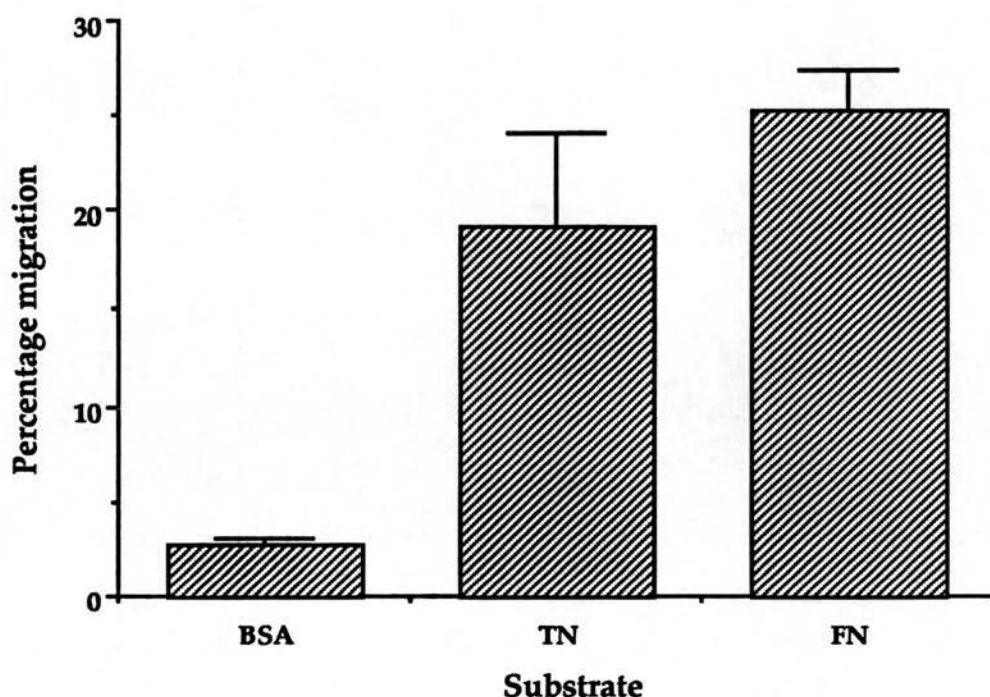


Figure 5.8 Percentage migration of SKOV-3 cells

This figure illustrates the mean percentage of cell migration from triplicate wells.

The data in figure 5.8 shows that 2.8% ($\pm 0.3\%$) of the total number of cells migrated through transwells coated with BSA, while 19.2% ($\pm 4.9\%$) of the cells migrated on TN and migration on fibronectin was at a level of 25.3% ($\pm 2.1\%$). However, while it was observed in the SKOV-3 ovarian carcinoma cell line that fibronectin supports a higher level of motility compared to TN, this difference is not statistically significant. Both proteins promote significantly greater migration than was observed on BSA coated transwells ($p = <0.01$ by Student-Newman-Keuls multiple comparison test).

The level of migration was also investigated in the PEO1 ovarian carcinoma cell line.

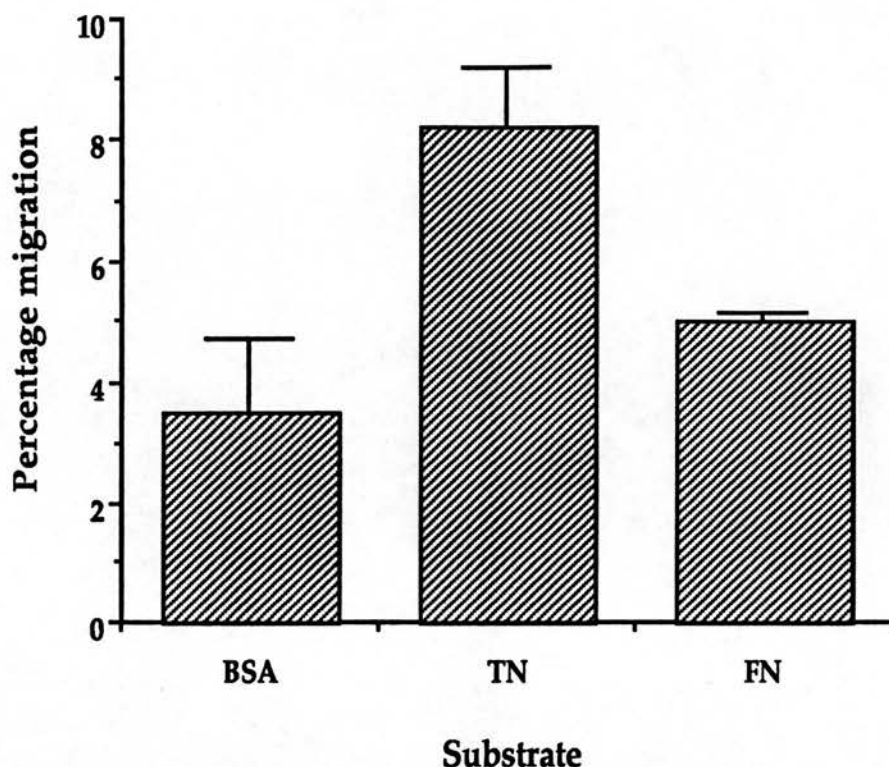


Figure 5.9 Percentage migration of PEO1 cells

The migration of PEO1 ovarian carcinoma cells was only measured on a single occasion. Each bar illustrates the mean percentage of migration from triplicate wells and the error bars represent standard deviation.

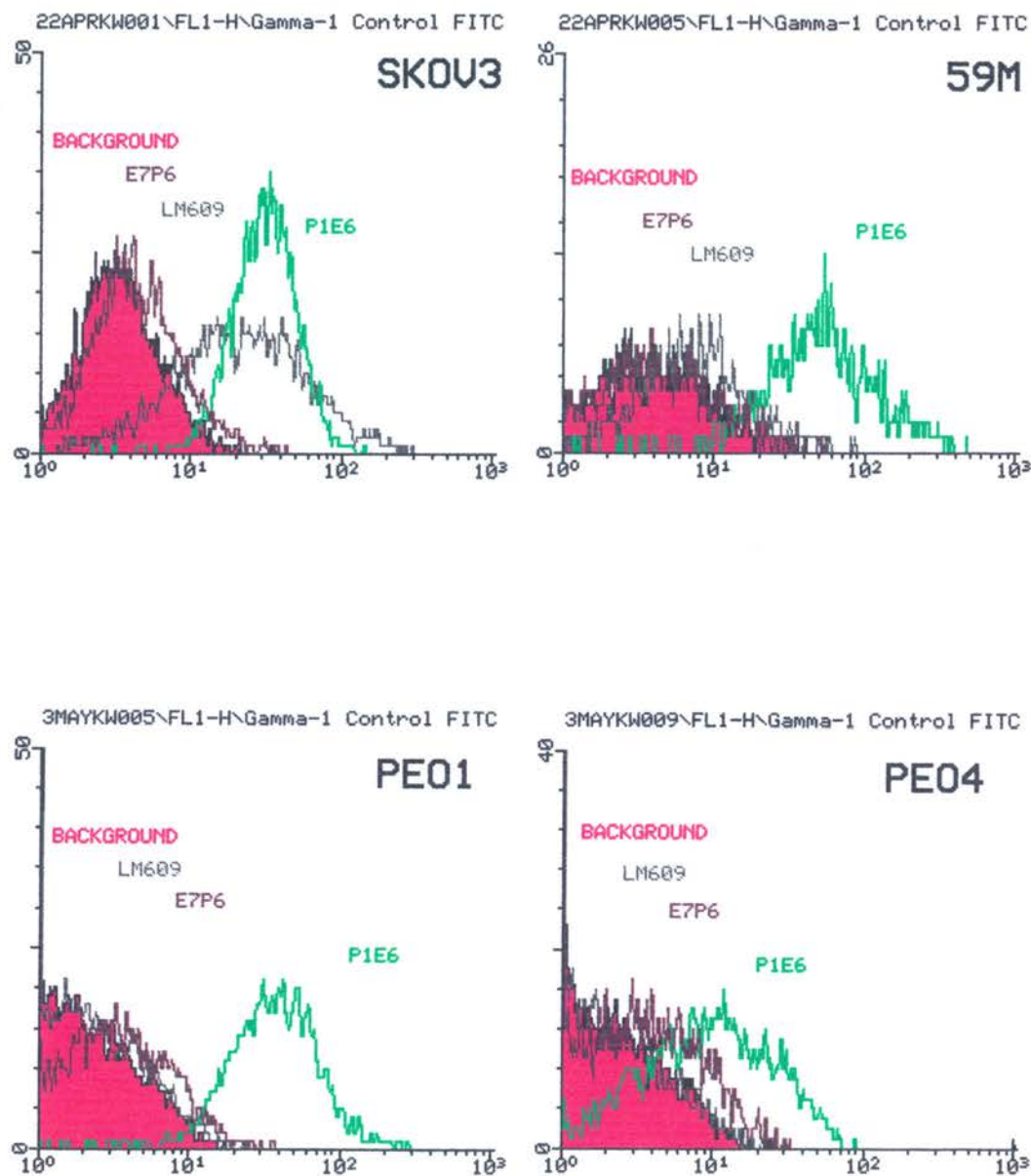
It was observed that both TN and fibronectin promoted migration over the level of BSA. TN was a more permissive substrate for PEO1 cell migration, with 8.1% ($\pm 0.9\%$) of the cells migrating through the membrane, compared with 5% ($\pm 0.2\%$) migrating on fibronectin. The level of migration on TN was significantly different to the levels of migration on fibronectin and BSA ($p = <0.05$ by Student-Newman-Keuls multiple comparison test). The general level of migration of this cell line was lower than that found in the SKOV-3 cell line.

5.3 Analysis of integrin expression in ovarian carcinoma cell lines

Integrins are cell surface receptors, which exist as heterodimers consisting of an α and β subunits. Tenascin has been demonstrated to bind to the integrins $\alpha 2\beta 1$, $\alpha 8\beta 1$, $\alpha 9\beta 1$, $\alpha V\beta 3$ and $\alpha V\beta 6$. Antibodies were available against $\alpha 2\beta 1$ (P1E6), $\alpha V\beta 3$ (LM609) and $\alpha V\beta 6$ (E7P6). The antibody stock solutions were diluted in PBS containing serum (P1E6 diluted 1:100, LM609 diluted 1:200 and E7P6 diluted 1:10). The antibodies and their recommended dilutions were provided by Dr J. Marshall, Richard Dimbleby Department of Cancer Research, St Thomas' Hospital, London. The ovarian cell lines were incubated with these antibodies and analysed by flow cytometry.

Figure 5.10

Flow cytometry analysis of ovarian cell lines using antibodies to integrins
This figure shows a typical plot of fluorescence versus cell number for each of the four cell lines



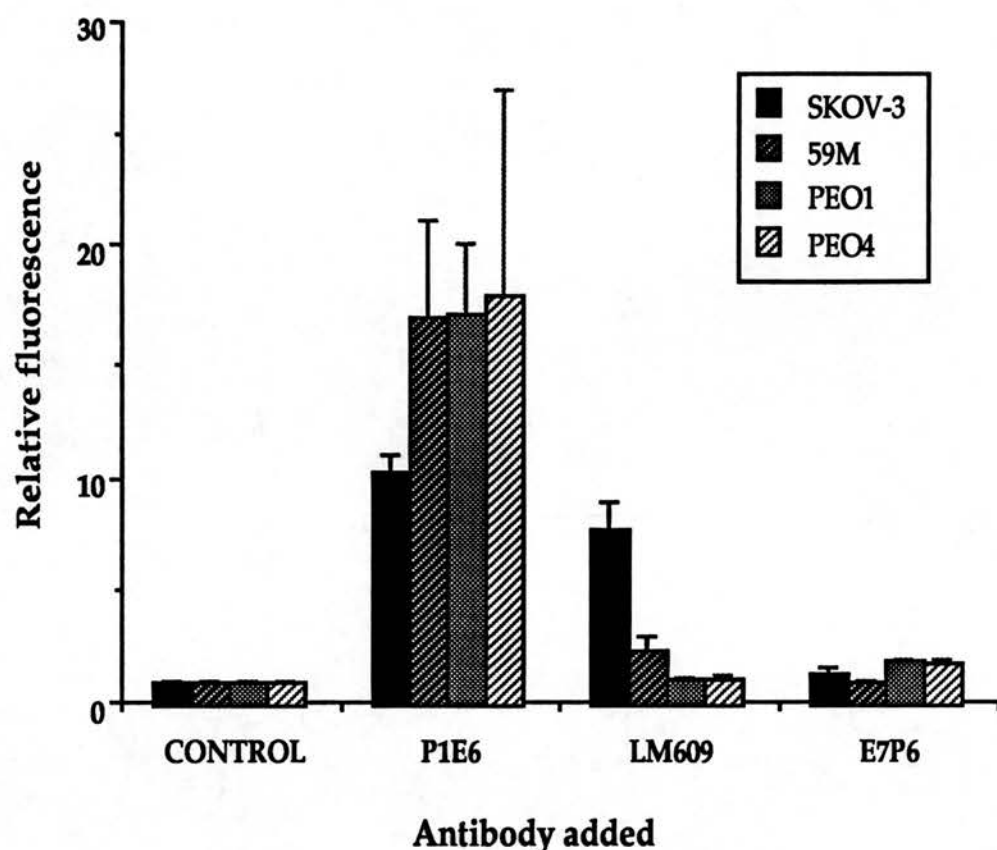


Figure 5.11 Profile of integrin expression in ovarian carcinoma cell lines

The median fluorescence of each cell line was assayed on two separate occasions and the relative intensity was calculated by comparison with the control level (control = 1.0 relative fluorescence unit). The bars in this graph represent the mean relative fluorescence of the two experiments with the error bars representing standard deviation.

It was observed that all 4 cell lines reacted with the antibody P1E6; this demonstrated that they all expressed the integrin $\alpha 2\beta 1$ on their cell surface. The SKOV-3 cell line also reacted with the LM609 antibody which is specific for the integrin $\alpha V\beta 3$. PEO1 and PEO4 cells did not react with this antibody, while the 59M cell line only showed a level of fluorescence which was slightly elevated when compared to control levels. None of the cell lines reacted with the E7P6 antibody and therefore they probably do not express the integrin $\alpha V\beta 6$.

5.4 Effects of tenascin on proliferation of an ovarian carcinoma cell line.

Studies in other cell lines have demonstrated that TN can stimulate growth (End *et al.* 1992). It was also observed that in co-culturing experiments (described in section 4.4.1) the number of PEO1 carcinoma cells in the mixed populations was greater than in those cultured alone.

Table 5.1 Changes in PEO1 cell numbers on co-culturing with fibroblasts

condition	PEO1 alone	PEO1 with PEO12	PEO1 with PEO9
cell number	1.8×10^6	4.8×10^6	4.025×10^6
(\pm s.d.)	(0.29×10^6)	(1.1×10^6)	(1×10^6)

These cells had been cultured with fibroblasts, in serum free conditions, for 48 h and in some cases the cell numbers were double that observed in the separate population. This experiment was designed to examine whether the levels of TN secreted by the fibroblast cells could be causing this increase in cell number.

TN was added to PEO1 cells at a concentration of $0.1 \mu\text{g/ml}$, the level secreted by PEO12 fibroblasts, and at a 10 fold increased concentration ($1 \mu\text{g/ml}$), to take into account the increases that may be found in the co-culturing experiments due to local increases in concentration. EGF (10^{-10} M) was used as a positive control and the control cells were grown in serum free conditions without any additions of factors. Media were changed and fresh factors were added on days 0 and 3. Cells were counted on days 0, 3 and 5.

The results from a typical growth experiment are illustrated in figure 5.12

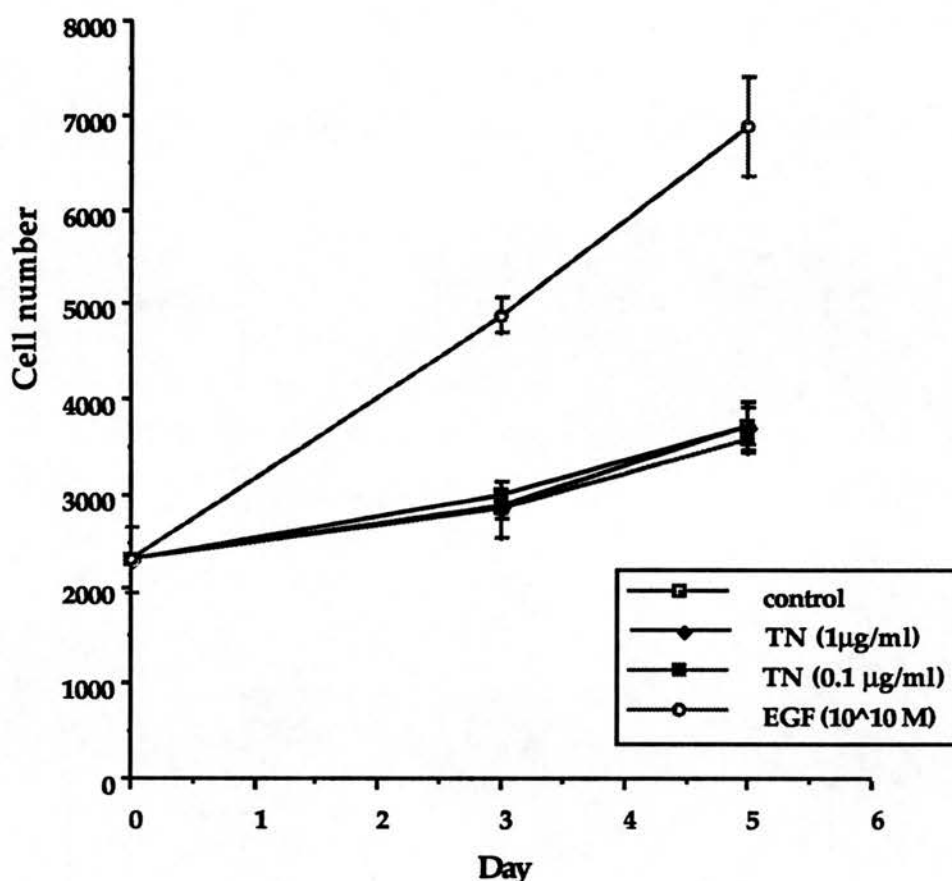


Figure 5.12 Effects of TN and EGF on the growth of PEO1 cells.

The points on this graph represent the mean counts from quadruplicate wells, error bars illustrate standard deviation. This experiment was subsequently repeated and produced consistent results.

As expected EGF stimulated the growth of PEO1 cells. In comparison with control levels, neither concentration of TN altered growth rate. This indicated that the TN secreted by the fibroblasts cells was unlikely to alter the growth rate of PEO1 ovarian carcinoma cells in the co-culturing experiments.

6.1 General expression of tenascin

This thesis includes studies on clinically derived materials from ovarian tumours which represent the first in which TN expression was observed, at the level of protein and RNA, in a series of ovarian tumours. Differences were observed in the pattern of immunohistochemical staining and species of RNA expressed, between malignant and benign tumours.

In 26 of the 32 malignant tumours, diffuse TN staining was detected throughout the tumour stroma. This pattern of staining was markedly different to that observed in normal ovarian tissue and the benign ovarian tumours. The majority of those malignant tumours showing this diffuse stromal staining pattern, expressed moderate or strong levels of TN. The highest intensities of stromal staining observed were found within this group of malignant tumours.

A group of 7 borderline tumours were studied and 4 of these demonstrated the diffuse stromal staining. This incidence of stromal TN expression was significantly higher than that in benign tumours. Whilst the distribution pattern in borderline tumours resembled that found in malignant tumours, the level of intensity was generally lower in the borderline samples with weak staining observed in 3 of the 4 tumours with positive stroma.

Of the 11 benign tumours studied, TN staining was undetectable in 2 samples and 8 showed a pattern of protein expression which was similar to that observed in normal ovary, with TN limited to focal expression around blood vessels. The presence of TN in the walls of blood vessels in

normal ovarian tissue was also noted by Tamura *et al.* (1993). TN has also been shown to be a vascular component in other normal tissues (Mackie *et al.*, 1992). Tamura *et al.* also demonstrated TN staining in ovarian structures such as regressing corpora lutea and atretic follicles; the sample of normal ovary examined in this thesis contained none of these structures. The benign tumour which demonstrated a moderate level of diffuse stromal staining was classified as having a mixed histology, consisting of cystadenofibroma/thecoma/granulosa.

These findings are comparable with those made in other solid tumours. In prostate tissue, Ibrahim *et al.* (1993) found that normal adult tissue showed only weak focal staining; they utilised the staining of blood vessels as an internal control. 80% of the benign tumours they studied expressed TN, the majority of this staining was focal (the TN was expressed as discontinuous periglandular bands). Of the malignant tumours examined 96% were positive for TN with high grade tumours demonstrating staining of the ECM surrounding tumour cells. Although their findings were similar to those observed in this study of ovarian tumours the pattern in malignant prostate tumours did not differ significantly from that in benign lesions. The findings within this thesis are, however, consistent with those made in endometrial tumours by Vollmer *et al.* (1990). They too demonstrated that in normal and benign tissue TN expression was weak and focal, whilst in endometrial adenocarcinoma the entire ECM of the stromal mesenchyme displayed diffuse TN staining.

Omental metastases are formed as the primary ovarian tumours shed cells into the peritoneal cavity; these implant on the mesothelium covering the omentum, invade the tissue and form new stroma and extracellular matrix. It was observed that omental metastases expressed a strikingly similar pattern of TN expression to their primary counterparts. Sections of normal omentum were investigated but no TN staining was observed. However these sections were of very poor quality due to the

difficulty in cutting the essentially fatty tissue. Ibrahim's study of prostate cancer also examined metastases in lymph nodes, and demonstrated that the stroma in these tumours stained intensely for TN. These data demonstrate that the new stroma which is generated when carcinoma cells invade a secondary tissue is also rich in TN, thus implying that TN may be involved in the metastatic process.

The overexpression of TN in the stroma of malignant ovarian tumours prompted further studies utilising ovarian cell lines to address the nature of the cell types which may be capable of producing TN.

Measurements of TN secretion, by ELISA, demonstrated that ovarian fibroblasts secrete TN into their media, while ovarian carcinoma cell lines do not secrete measurable levels of TN. Several other studies have demonstrated that fibroblasts are capable of producing TN (Inaguma *et al.*, 1988; Chiquet-Ehrismann *et al.*, 1989). However an equal number of studies have demonstrated that epithelial cells are also able to produce the protein (Herlyn *et al.*, 1991; Kawakatsu *et al.*, 1992; Lightner *et al.*, 1994). Indeed Chiquet-Ehrismann (1995) states that "it was believed that epithelial cells do not synthesise TN, whereas fibroblastic cells do. This prejudice is certainly false... it remains to be seen whether almost all cells may be capable of TN production given the appropriate environment and stimuli." Most of the epithelial cell lines studied required addition of growth factors or serum in order to produce TN; melanoma cell lines are capable of producing TN under growth factor-free culturing conditions (Herlyn *et al.*, 1991), indicating constitutive expression of TN. These studies have shown that glioma and melanoma cell lines are the only epithelial cells capable of producing levels of TN comparable to that found in fibroblasts. Glioma cells secrete 5-10 $\mu\text{g/ml}$ (Erickson and Lightner, 1988) fibroblasts have been shown to secrete levels between 200-500 ng/ml (Erickson and Bourdon, 1989).

This study demonstrated that fibroblasts are capable of secreting levels

over 100 fold higher than the ovarian carcinoma cells *in vitro*. The levels measured were comparable to those found by Erickson and Bourdon (1989). The TN measured was secreted into the media and it is likely that intracellular and matrix TN were not measured. Attempts were made to measure TN in cell lysates, however, these were unsuccessful as large amounts of non-specific background antibody binding were observed; this was not overcome by adding blocking stages to the ELISA protocol, or using different immulon trays. Carnemolla *et al.* (1992) demonstrated that transformed cultured cells were unable to deposit TN into the matrix; any protein they produced was secreted into the media. Conversely untransformed fibroblasts retained 60-90% of their TN in the matrix. These observations implied that the values of secretion of TN obtained for the ovarian fibroblasts may be an underestimate of the true value, whilst any TN produced by the carcinoma cells should have been secreted into the media and therefore detected by the assay. This indicates that for carcinoma cells to produce amounts of TN comparable to those from fibroblasts there would have to be between a 100 fold and 1000 fold induction of synthesis.

Within this thesis levels of TN secretion were investigated in the breast carcinoma cell lines MDA-MB-231, T47D, MCF7 and ZR-75-1. The only cell line to secrete detectable levels of TN was MDA-MB-231, which secreted 8.7 ng/10⁶ cells/ml. Lightner *et al.* (1994) assayed culture supernatant from breast carcinoma cell lines by ELISA. The results obtained in this study were compared to those found by Lightner, to examine whether there was consistency between the two different ELISA methodologies. Lightner *et al.* (1994) examined 11 breast carcinoma cell lines and found that the MDA-MB-231 cell line was 1 of 4 breast carcinoma cell lines which secreted detectable TN; 7 other cell lines they examined, including T47D, MCF7 and ZR-75-1, did not secrete TN. The level of TN they observed from the MDA-MB-231 cells (89 ng/ml) was 10 fold greater than that observed in this study. This difference is probably explained by the presence of serum in the media used by Lightner's

group, serum is known to be capable of stimulating TN synthesis. The antibody used by Lightner had been determined not to cross-react with bovine TN whereas experiments indicated that the antibody used in this study did cross react with media containing serum. Kawakatsu *et al.* (1992) in a qualitative assay, also observed that the MDA-MB-231 cells produced immunoprecipitable TN, while the other three cell lines did not produce any TN; their assay was carried out in serum-free conditions.

The observations on TN expression made in the clinical specimens, at the level of protein, were also consistent with the presence of TN RNA. RT-PCR detected TN RNA in all the tumours examined. Multiple isoforms were detected in the samples including the products which had been previously described by Siri *et al.* (1991). The forms described by Siri had been fully characterised by sequencing and probing with oligonucleotides with specificity equivalent to oligonucleotides 5 and B, as used in this study; these oligonucleotides hybridised with bands containing FN type III repeats 5 and B. The additional oligonucleotides A4 and C confirmed the description of the FN type III repeats present in the larger isoforms as described by Siri *et al.* (1991).

Although all samples examined expressed TN RNA, differences were seen in the RNA splicing patterns between malignant and benign tumours. All but 1 of the 6 benign tumours examined produced a pattern similar to that found in normal ovary. In malignant tissue there was an increased expression, particularly of the ~490 and 556 bp products. Paired samples of omental metastases were not examined as the fatty nature of the tissue prevented efficient RNA extraction.

The band observed at ~490 bp had not been previously described by Siri *et al.* (1991). This band hybridised with specific oligonucleotides recognising FN type III repeats 5 and B. The molecular weight of the band is not sufficient for it to contain the complete form of both these repeats; this indicates that it may be produced by internal splicing sites, or may contain

a different FN type III repeat with sequence similarities to repeats 5 or B. Sriramarao and Bourdon (1993) have described the presence of an additional FN type III repeat. The possibility that this repeat could be responsible for the ~490 bp band was not directly investigated, however, several points indicate that this is probably not the case. Firstly the molecular weight of a form containing repeats 5 and AD1 would only be 260 bp. Secondly Siri found that this repeat was either spliced out alone or with repeats C and D, they never observed forms where it was included alone. Their group also found that fibroblasts did not produce isoforms containing this repeat, and as is described below ovarian fibroblasts do produce the ~490 bp form. The most convincing piece of evidence is that the specific oligonucleotide B hybridises with this band, the sequence of oligonucleotide B is not found in repeat AD1. It thus appears that the FN type III repeat AD1 is not responsible for the ~490 bp band, however, the presence of this repeat may account for some of the other intermediate forms, e.g. the ~750 bp form, which were not described by Siri *et al.* (1991) but were detected in the ovarian tumour samples.

Most other studies have demonstrated, using northern blotting techniques, that the larger form of TN is preferentially expressed in malignant tumours (Borsi *et al.*, 1992). This larger form is generally presumed to be the full length transcript of TN. Borsi *et al.* (1992) also showed that carcinoma cell lines were capable of producing intermediate molecular weight forms of TN. This could provide an explanation for the obvious differences observed in this study, in the expression of intermediate molecular weight RNA splice variants, between malignant and benign ovarian tumours.

RT-PCR was used to detect TN RNA in samples of total cellular RNA prepared from cell lines. The cells were cultured in medium containing serum. Serum is a known inducer of TN synthesis, therefore this may

account for discrepancies between the observations made on the levels of "basal" TN protein expression and the detection of TN RNA. Cells were cultured in serum containing media, as confluent cells were required for efficient RNA extraction.

A panel of 9 ovarian carcinoma cell lines were examined and multiple forms of TN RNA were detected in all these samples. The ovarian fibroblasts PEO12 and PEO27 also demonstrated the presence of TN RNA. Not all cell lines tested were positive for TN RNA, the breast carcinoma cell lines ZR-75-1, T47D and MCF7 were negative.

It was observed that the fibroblasts produced a pattern of bands which was very similar to that observed in the malignant tumour samples. This provides further evidence to support the theory that the overexpression of TN in malignant ovarian tumours may be due synthesis of the protein by ovarian fibroblasts. Many of the bands observed in the carcinoma cell line samples were not found in these primary tumours. This indicates that while ovarian carcinoma cells are capable of synthesising TN RNA they are not responsible for producing the RNA isoforms observed in the malignant tumours. This may be because they may not have the correct stimuli *in vivo* to produce TN.

Which of these RNA variants may result in the expression of proteins is unknown and further studies would be required to characterise this. While any internal alternative splicing should be capable of producing TN protein it is possible that secondary structures of the RNA may differ between isoforms resulting in some forms not being translated.

The staining observed in the sections of malignant tumours demonstrated that all the TN was deposited within the stroma. Experiments measuring the levels of TN secretion in cell lines indicated that ovarian fibroblasts are probably the major source of this TN. It should be noted that experiments in other cell types have demonstrated that epithelial cells are capable of producing TN under certain circumstances (Herlyn *et al.*, 1991; Kawakatsu *et al.*, 1992; Lightner *et al.*,

1994). RT-PCR data indicates that the ovarian cell lines tested are capable of producing multiple isoforms of TN RNA. However, within the tumour samples examined no epithelial cell staining was observed; in sections of breast tumours stained with the same antibody a small number of tumours did show tumour cell staining, this phenomenon has previously been demonstrated by Ishihara *et al.* (1995). The data within this study suggests that fibroblasts are the major producers of TN in ovarian tumours. The identity of the cell lines producing TN in ovarian tumours could be definitively identified by in situ hybridisation. Yoshida *et al.* (1995) proposed myofibroblasts as a candidate for the producers of TN in breast tumour stroma. These cells resemble smooth muscle cells rather than fibroblasts; fibroblasts cultured in the presence of TGF- β differentiate into myofibroblasts and concurrently begin to secrete increased levels of TN.

The expression of TN is complicated by the fact that it can exist as different isoforms. The specificity of the antibody, used in the immunohistochemical studies and ELISA, is unknown. The antibody was raised against TN secreted by the U251 glioma cell line, which is known to secrete both large and small isoforms of TN, however, whether both forms can be recognised by this antibody has not been reported. A study by Zagzag *et al.* (1996) utilised the same monoclonal antibody to immunoprecipitate TN, subsequent detection of the precipitated protein used a different antibody, both large and small TN isoforms were detected in the samples. While this experiment is still not absolute proof of the specificity of the monoclonal antibody, the detection of both the large and small isoforms implies that the monoclonal antibody used in their study, and this thesis, can recognise both forms of the protein.

The possibility that the alternatively spliced isoforms of TN may have different functions is discussed in section 6.4.

The existence of other TN family members has recently been discovered.

Again it is unknown whether this antibody cross reacts with other TN proteins or is specific for TN-C. TN-R has not been identified in humans and its distribution in chicken and rat is limited to the nervous system. Human TN-X does have sections of homology with TN-C, however the FN type III repeats for example are more homologous to fibronectin than to TN-C. TN-X also has a distribution pattern which is distinct and often reciprocal to that of TN-C, although they are co-expressed in blood vessels (Matsumoto *et al.*, 1994). The likelihood is that the antibody is specific, and the protein detected in this study is TN-C.

6.2 Control of tenascin secretion

In all the tumours examined, the immunoreactivity was strongest at epithelial-mesenchymal junctions (as shown in figure 3.2). This observation could have a number of different explanations: (1) the TN may be accumulating in the basement membrane of these regions, as may be expected with an ECM protein; (2) tumour cells could themselves be producing the TN and depositing it in the matrix; or (3) the fibroblasts in malignant tumours may be inherently different, from those in benign tumours, in their capacity to express TN. The expression data from this study of ovarian tumours and cell lines indicates that fibroblasts are likely to be responsible for the synthesis of TN. Whether these fibroblasts from malignant sources are inherently different was not investigated, however this is unlikely, as the genetic changes producing the tumours examined had occurred in the epithelial cells to produce an epithelial tumour.

Another explanation, with which this pattern is consistent, is the hypothesis that TN expression is under paracrine control. The investigations into general expression examined which cell types were most likely to be responsible for the expression of TN *in vivo*.

Evidence for the paracrine stimulation of TN secretion has been demonstrated in several tissues. In an elegant experiment Inaguma *et al.* (1988) cultured embryonic mammary epithelium on an embryonic mammary fat pad and observed a "halo" of TN around the growing epithelium. Julian *et al.* (1994) noted a similar phenomenon in cultures of uterine stroma and epithelia.

The experiments in this study, in which ovarian fibroblast and epithelial cells were co-cultured maintained a physical separation of the two cell types while allowing the circulation of any secreted factors. It was observed that the mixed populations secreted increased amounts of TN. These findings were consistent with the hypothesis that the expression of TN is controlled by diffusible paracrine factors. These data agree with findings by Chiquet-Ehrismann *et al.* (1989), who demonstrated that

conditioned media from MCF-7 epithelial cells stimulated the production of TN by fibroblasts.

6.2.1 Growth factor regulation of tenascin expression

A number of growth factors and cytokines and hormones have been implicated in the control of TN, however, this is the first study to examine potential modulating factors in ovarian systems. Initially the immunohistochemical data from the malignant ovarian tumours was examined to elucidate relationships between TN staining and the expression of growth factors and steroid hormone receptors. Subsequent experiments utilised cell lines in order to investigate, *in vivo*, modulations by growth factors, hormones and cytokines.

The growth factor TGF- β was one of the first modulators of TN to be identified (Pearson *et al.*, 1988). TGF- β has been shown to play a role in normal ovarian function, however little is known of the expression and effects of TGF- β in ovarian cancer (Bartlett *et al.*, 1992). Data were available on the expression of TGF β mRNAs in a cohort of the ovarian tumours used within this study, however there was no data on TGF- β protein. A significant positive correlation was found with TN protein expression in the malignant tumours and expression of TGF- β_1 mRNA. No correlation was observed with TGF- β_2 or β_3 . This is consistent with findings made by Walker *et al.* (1994) in breast tumours, which demonstrated a relationship between strong expression of TN and TGF- β_1 , both measured immunohistochemically.

The relationship observed between strong TN staining and expression of TGF- β_1 in the malignant tumours suggested that this growth factor plays an important role in the regulation of TN production in ovarian cancer. Further evidence for this theory was also provided by the dose dependent induction of TN secretion in ovarian fibroblasts. The maximum induction of TN was observed upon addition of 10 ng/ml TGF- β_1 . This result was comparable to findings by Shrestha *et al.*, (1996), who also

demonstrated a dose dependent induction of TN in mouse fibroblasts, with the effect reaching a maximum at 10 ng/ml TGF- β_1 .

It has been demonstrated that the ovarian carcinoma cell line PEO1 can produce TGF- β_1 mRNA, however, whether these cells produce functional TGF- β_1 protein has not been proven. This data indicates that one of the factors responsible for the stimulation of TN secretion in co-culturing experiments may be TGF- β_1 ; this could be tested by using antibody to neutralise TGF- β_1 in the medium, as described by Chiquet-Ehrismann *et al.* (1989). There is also data to indicate that TGF- β_1 may alter the proportions of different splice variants. Zhao and Young (1995), demonstrated that TGF- β_1 preferentially stimulated the synthesis of the smaller TN isoform in rat lung cells.

The relationship between TN staining in malignant tumours and the expression mRNA for the growth factors EGF and TGF- α , or the EGF receptor was investigated; no correlation with the level of TN staining was observed. These factors were not investigated by immunohistochemistry, the RT-PCR data on these factors was not quantitative and investigations examining the level and location of protein expression may have revealed a significant relationship.

The effects of a number of growth factors, hormones and cytokines were examined in ovarian fibroblasts. The largest stimulation of TN secretion in these cells was produced by TGF- β_1 , but IGF-II also produced a small yet consistent increase in the levels of TN secreted by the ovarian fibroblasts. TGF- α , IGF-I, endothelin and EGF were shown to have no effect on the level of secretion from these cells. Interferon- γ inhibited the secretion of TN by the fibroblast cell line. This is in contrast to observations made by Harkonen *et al.* (1995) who demonstrated induction of TN secretion by interferon in lung epithelial cells. Effects of regulating factors on TN secretion have been found to vary between different cell types (Rettig *et al.*, 1994) and interferon has been shown to inhibit the synthesis of other

As with most other proteins which have been postulated to be "tumour markers" once more detailed studies were undertaken, the distribution of TN was found to be much more widespread than originally thought. In normal tissues the expression of TN is highly regulated leading to its restricted distribution pattern. The overexpression of TN in tumours is likely to be due to the loss of this regulation, however it is also possible that the malignant cells could be expressing the protein.

The high expression of TN at epithelial-mesenchymal junctions is consistent with the paracrine induction of TN synthesis, experiments co-culturing populations of fibroblasts and carcinoma cells indicated that a paracrine loop exists in ovarian cell systems. The regulation of TN synthesis, observed in normal tissues, is potentially overcome in tumours by the constitutive overexpression of growth factors. Data has shown that ovarian carcinoma cells can overexpress a number of paracrine factors including TGF- β_1 (Bartlett *et al.*, 1992). A potential model of TN expression resulting from interactions between epithelial and mesenchymal cells is illustrated in figure 6.1.

The TN promoter has been shown to have response elements which are potential targets for transcription factors implicated in growth factor signalling pathways, notably the phorbol ester response elements TRE/AP-1 (Jones *et al.*, 1992). TGF- β is known to act through transcription factors capable of binding these sites (Barcellos-Hoff, 1993).

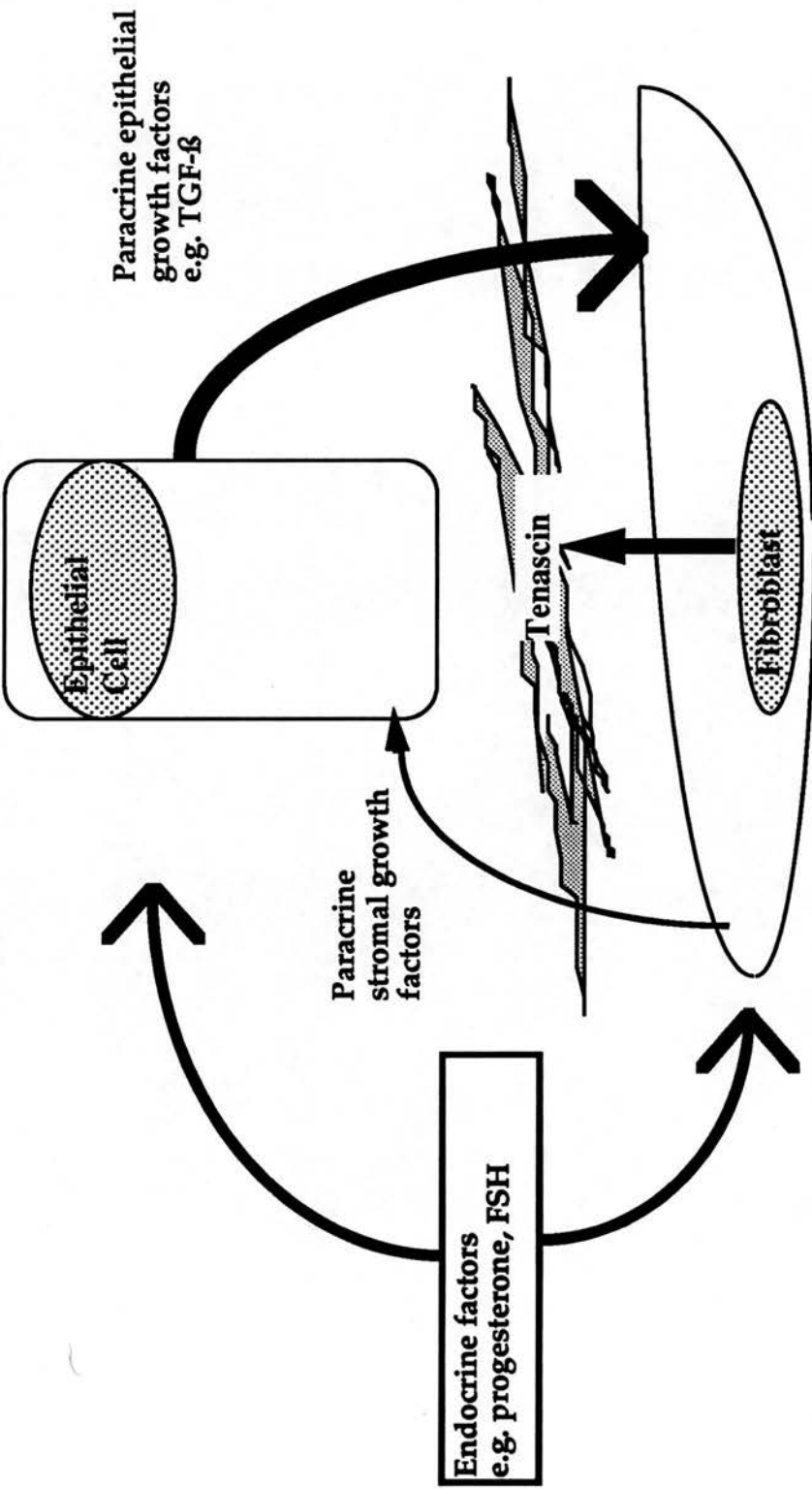


Figure 6.1 Potential modulation of TN secretion in ovarian systems

This schematic diagram illustrates the potential paracrine and endocrine loops which may influence the secretion of TN by ovarian fibroblasts.

6.2.2 Hormonal regulation of TN secretion

Addition of progesterone to ovarian fibroblasts produced a small but consistent stimulation of TN secretion. The promoter of TN has been studied (Jones *et al.*, 1990; Jones *et al.*, 1992; Gherzi *et al.*, 1995) but a known hormone response element has not yet been identified; this makes it a possibility that these hormones do not act directly upon the promoter. An examination of oestrogen and progesterone receptor expressed in the malignant ovarian tumours revealed that there was no significant relationship between TN staining and the levels of these receptors. It is possible that the increase in TN observed in the cultured fibroblasts may be due to progesterone stimulating the production of growth factors by the fibroblasts themselves resulting in an autocrine stimulation of TN secretion. No stimulation of TN secretion by the cultured fibroblasts was observed upon addition of oestrogen. The gonadotrophins FSH and HCG inhibited the secretion of TN. These hormonal modulations of TN secretion in fibroblast cells are surprising as it is generally assumed that the targets of these hormones are ovarian epithelial cells. However, the presence of progesterone receptor on ovarian fibroblasts has been reported (Press and Greene, 1988). Additionally, sections of ovarian tumours, stained for progesterone receptor, were examined in the present study (data not shown); weak nuclear staining was detected in the stromal tissue.

The pattern of expression observed by Tamura *et al* (1992) in normal cycling ovaries, is consistent with the data in section 4.3.2. These data demonstrated that TN secretion in ovarian fibroblasts can be influenced by the hormones progesterone, follicle stimulating hormone and human chorio gonadotrophin. Tamura *et al* found that expression of TN varied according to the stage of the menstrual cycle. No significant TN staining was observed in preovulatory follicles. In the early luteal phase, distinct TN staining was seen at epithelial mesenchymal boundaries and during involution of the corpora lutea diffuse TN staining was observed.

Tamura *et al.* (1992) suggest that these alterations in TN secretion are due to stromal-parenchymal interactions. Their data is also consistent with observations made in other hormone dependent tissues and data presented within this study demonstrating that progesterone can stimulate and FSH inhibit TN secretion, in ovarian fibroblasts. Therefore, it is possible that the expression of TN in ovaries may also be regulated, in part, in an endocrine manner. If the levels of the hormones progesterone and FSH are plotted throughout the menstrual cycle it can be observed that the peak in TN expression corresponds to the time of the cycle when progesterone levels are high and FSH levels are low (figure 6.2). Progesterone is produced by the corpus luteum and it was within regressing corpus lutea that Tamura *et al.* (1992) observed the strongest TN staining.

Further investigations of the hormonal control of TN, would be required to confirm these theories; for example it would be of interest to observe whether progesterone is capable of reversing the inhibition of TN secretion produced by FSH, and time course experiments may provide some indication of whether the hormonal control is direct or involves the induction of other factors.

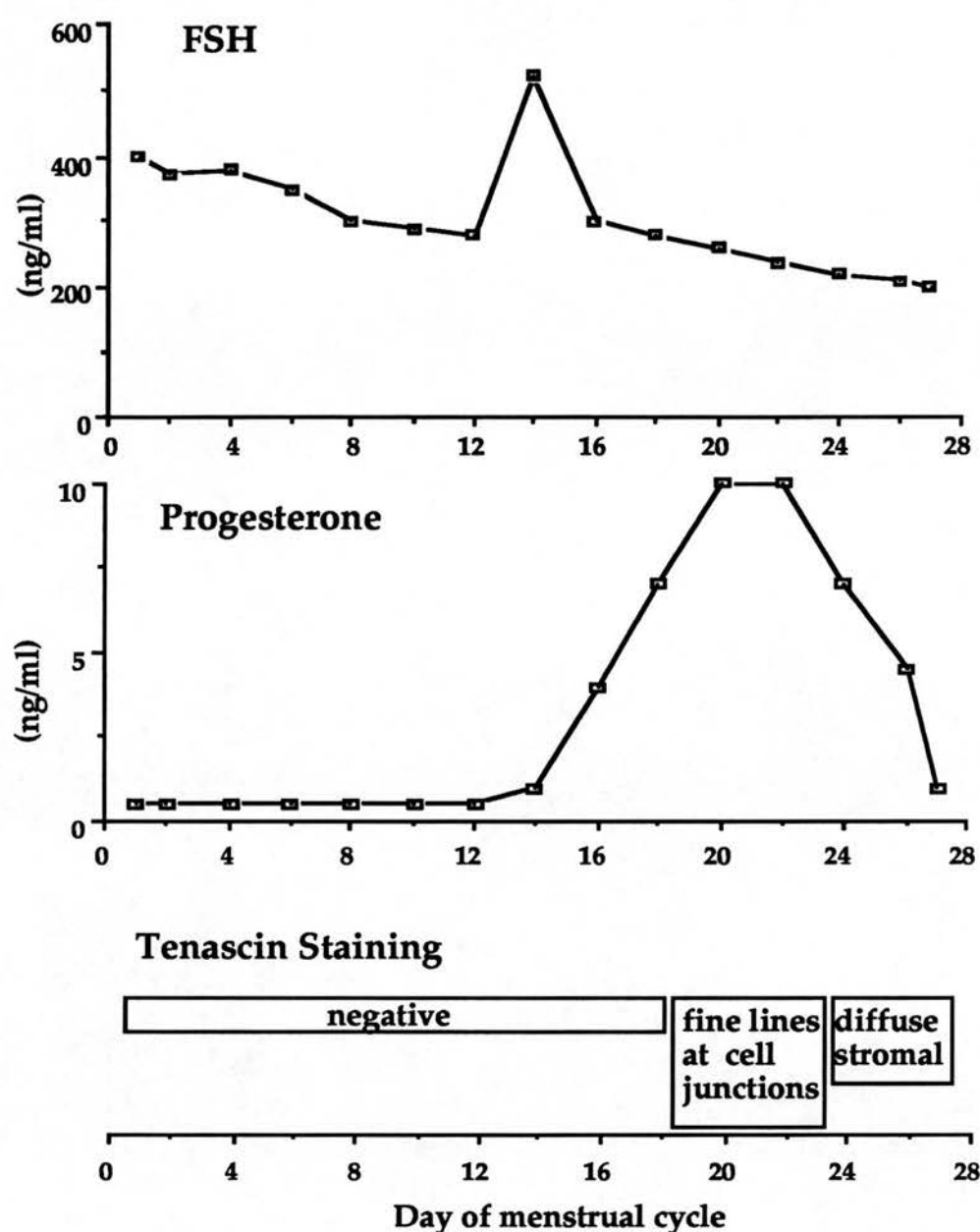


Figure 6.2 Simplified illustration of hormone levels and tenascin expression in normal ovary

Mean plasma levels of FSH and progesterone were taken from Rang and Dale (1991). Day 1 is the onset of menstruation and ovulation occurs on day 14. The description of TN staining was adapted from Tamura *et al.* (1993).

Ferguson *et al.* (1990) made similar observations regarding the variable expression of TN in normal breast tissue throughout the menstrual cycle; the peak in TN expression again coincides with the peak in progesterone levels. It was also observed that TN levels in the uterus varied throughout the menstrual cycle and in ovariectomized mice the expression of TN was down regulated; the level could be restored by progesterone but not oestrogen. This is compatible with the findings reported here in ovarian fibroblasts, where a small induction of TN secretion was observed upon addition of progesterone, while oestrogen produced no effect on TN levels.

6.3 Functional properties of tenascin expression

The functions of TN in general are still the subject of much debate. The protein has been demonstrated to influence many aspects of cells, effects have often been shown to be contradictory and cell specific. This is the first study to examine the interactions of TN with ovarian cell lines.

The reported functions of TN in other tissues and its overexpression in malignant and highly invasive tumours has led to speculation that the protein may be involved in the processes of invasion and metastasis. This study examined the interactions of ovarian carcinoma cells with TN in relation to two key components of the metastatic process; namely adhesion and migration.

The use of adhesion assays demonstrated that TN promotes adhesion of ovarian carcinoma cell lines to tissue culture plastics. Differences were observed between the adherence of the cell lines to TN, compared with fibronectin and collagen IV. SKOV-3 and 59M cells attached to TN at a lower level than collagen IV and fibronectin. PEO1 cells attached more strongly to TN than to collagen IV, and PEO4, which showed a

preferential level of attachment to TN than fibronectin. Further studies should examine the interaction of TN with the other ECM proteins in particular fibronectin. TN has been shown to interfere with the cell binding properties of fibronectin (Chiquet-Ehrismann *et al.*, 1988); this may be through competition for cellular receptors.

Berens *et al.* (1996) observed that adhesion of glioma cells to TN increased to a maximum level at 10 μ g/ml of protein, but adhesion decreased at concentrations of 33 and 100 μ g/ml. Experiments in this study with SKOV-3 cells demonstrated that adhesion to TN did increase to a maximum at 10 μ g/ml, however it was observed that the level of adhesion plateaued and no decline was noted in this cell line even when taken out to 40 μ g/ml.

Observations made by examining the cells under phase contrast microscopy were generally consistent, in terms of apparent levels of attachment, with the quantitative measurements. The majority of other studies of epithelial cells have demonstrated that TN inhibits cell spreading and most cells have a more rounded morphology when compared with their appearance on other ECM substrates (Kawakatsu *et al.*, 1992). Within this study examination of PEO4 and PEO1 cells revealed that they did not spread on TN and remained rounded; no such marked differences were observed with the 59M cell line.

The observation of the SKOV-3 cell line demonstrated that the cells adopted a "spindly" appearance on TN, with the apparent formation of dendritic projections. This can be compared to the study by Canfield and Schor (1995); they demonstrated that TN mediated the "sprouting" of endothelial cells. Endothelial cells which are forming projections are more angiogenic. These sprouting cells are very similar in appearance to the SKOV-3 cells cultured on TN. Whether this morphological difference is implicated in the invasive behaviour of SKOV-3 remains to be elucidated; matrigel based invasion assays would provide a useful tool for further studies.

Examination of the effects of TN on migration demonstrated that TN promotes the migration of the SKOV-3 cell line over the level observed on BSA, however the level of motility on TN did not appear to be as large as it was on fibronectin. An experiment using the PEO1 cell line indicated that TN and fibronectin were also able to promote cell migration, and in this instance the level of migration on TN was greater on TN than fibronectin. Migration was assayed on a single concentration of TN (10 $\mu\text{g/ml}$), and therefore these data cannot be directly compared with those observations made by Berens *et al.* (1996). They observed the migration of glioma cells on TN and found levels increased on 1 and 3 $\mu\text{g/ml}$ TN but migration was actively suppressed on 30 or 100 $\mu\text{g/ml}$ TN. Other reports indicate that TN can affect cell motility and migration *in vivo*; for example the expression of the large TN variant correlates with cell migration in the developing cornea (Kaplony *et al.*, 1991). It has also been shown that TN inhibits migration of mesodermal cells during amphibian embryo gastrulation (Riou *et al.*, 1990)

TN has been demonstrated to interact with a number of integrins (Yokosaki and Sheppard, 1995). The expression of cellular adhesion molecules in ovarian cancer, such as integrins, have not been extensively studied. A recent study (Bridges *et al.*, 1995) demonstrated that in ovarian tumours there was an apparent up regulation of $\beta 1$ and $\alpha 2$ integrins while there was a loss of $\alpha 1$ chains. Gardner *et al.* (1995) examined the expression of $\beta 1$, $\beta 2$, $\beta 3$ and $\alpha 4$ integrins in a panel of 13 ovarian tumour cell lines (not including any of the lines in this study). They found that $\beta 1$ and $\beta 3$ integrins were expressed at high levels on the majority of these cell lines.

The flow cytometric analysis of integrins in this study, demonstrated that all of the cell lines expressed the $\alpha 2\beta 1$ integrin which is capable of binding to TN (Sriramarao *et al.*, 1993). The SKOV-3 cell line also expressed the integrin $\alpha V\beta 3$, this integrin has been shown to be capable of interacting

with TN (Sriramarao *et al.*, 1993) and is strongly implicated in the malignant behaviour of melanoma (Seftor *et al.*, 1992) and glioblastoma (Gladson and Cheresch, 1994). Seftor *et al.* (1992) demonstrated that activation of the $\alpha V\beta 3$ integrin resulted in enhanced invasiveness of melanoma cells. Berens *et al.* (1996) demonstrated that adhesion to TN could be blocked with anti- $\beta 1$ antibodies but was enhanced by incubation with anti- $\beta 3$ antibody.

Ramos *et al.* (1996) demonstrated that the integrin $\alpha V\beta 6$ is induced in squamous cell carcinoma and this integrin appeared to be preferentially expressed on poorly invasive cells. None of the cell lines examined in this study expressed this integrin, which has also been shown to be capable of binding TN. Carreiras *et al.* (1995) also demonstrated that the ovarian carcinoma cell line IGROV-1 did not express $\alpha V\beta 6$ but did express $\alpha 2\beta 1$ and $\alpha V\beta 3$.

Other integrins have been shown to interact with TN, antibodies were not available to these proteins. It is therefore possible that the ovarian cell lines may express any of these integrins in addition to those identified.

The experiments carried out on the PEO1 ovarian carcinoma cell line demonstrated that TN, at levels secreted by fibroblast cells, did not stimulate the growth of these cells. Adhesion assays and analysis of the integrin receptors indicated that these cells were capable of interacting with TN. This suggests that in ovarian systems TN may not play a significant role in proliferation and its function may be associated more with the invasive aspect of ovarian cancer.

Other studies, however, have shown that TN can stimulate the growth of quiescent mouse fibroblasts and conversely inhibit the growth of the epidermal cell line Pam212 (End *et al.*, 1992). In another study Crossin (1991) demonstrated that TN inhibited the growth of stimulated fibroblast cells. Although it has been speculated that the mitogenic effect of TN may

be due to contamination with growth factors, End *et al.* (1992) demonstrated that there was no increase in tyrosine phosphorylation upon addition of the TN, thus making it unlikely that a contaminating growth factor was responsible.

It has been demonstrated that TN expression is cell cycle dependent with a marked increase in synthesis and accumulation of the larger isoform of TN at the transition from G₀ to G₁ (Borsi *et al.*, 1994). This change in distribution of isoforms suggests that the two main isoforms may play different roles in cellular proliferation, potentially through modulating the level of cellular attachment to the substrate. However experiments carried out using different fragments of TN (End *et al.*, 1992) demonstrated that while the mitogenic activity was located within the FN type III repeats, both the splice variants tested could stimulate growth indicating that the mitogenic signal was not located in the alternatively spliced segments. These fragments were added to the media and it is possible that different isoforms may produce differing effects when they are part of the matrix.

The fact that different isoforms may possess different functions could explain the contrary results on proliferation, as both End and Crossin used different sources of TN to this study, and the discrepancies noted could be due to different isoform compositions of the TN sources used. However it is also probable that TN may exert differing effects on different cell types, as the protein has been shown to interact with a number of different receptors, the effects and expression of which may also vary.

6.4 The role of tenascin in ovarian cancer

It has been hypothesised that proteins may be expressed superfluously in tissues where they are non-functional, thus Erickson (1993) speculates that the high levels of TN observed in, for example healing wounds, may simply be an accident of gene regulation, tolerated because TN is an innocuous molecule in these situations. This study however provides

evidence that TN is capable of interacting with tumour cells, to promote adhesion, migration and alter cell morphology. These interactions imply that TN is a protein involved in the process of cancer progression. Its actual role in ovarian cancer can be speculated upon, from the data presented within this thesis.

The overexpression of TN in malignant tumours results in a stroma with a different composition of ECM proteins to that observed in benign tumours and normal ovary. The pattern of expression observed in the malignant tumours does, however, resemble that described by Tamura *et al.* (1992), who described a stronger, more diffuse expression of TN during regression of the corpora lutea. In normal ovary this stromal expression is transient and limited to the time of tissue reorganisation. The inappropriate expression of TN in the stroma of malignant tumours implies that it may be involved in the "reorganisation " of the tumour and its surrounding stroma, which results in the movement of the tumour cells from their original site.

Yoshida *et al.* (1995) speculated that TN may be capable of both promoting cancer cell invasion and protecting against invasion. In breast cancer high levels of TN expression have been found to correlate with poor prognosis (Ishihara *et al.*, 1995). This may be explained by the anti-adhesive properties of TN; helping cells detach from their substrate, decreasing focal adhesion and promoting migration. Conversely, in colon cancer it has been observed that high levels of TN in tumours results in lower levels of metastasis (Sugawara *et al.*, 1993). This protective effect may be due to TN creating a barrier around the malignant cells which they can not attach to.

These studies of ovarian cancer have revealed that there is overexpression of TN in malignant ovarian tumours. No relationship was observed between the degree of TN expression and clinical stage. Whilst clinical stage indicates the spread of the disease, all malignant tumours, by definition, are invasive. It is therefore unclear from this data

whether TN may be promoting or protecting against invasion in ovarian cancer. It was noted that whilst borderline tumours often expressed diffuse stromal TN, the intensity of staining was lower than that observed in malignant tumours. Borderline tumours have low metastatic potential and the fact that they demonstrate a level of staining which could be considered intermediate between benign and malignant suggests that the expression of TN increases in more invasive tumours.

The interaction of ovarian carcinoma cell lines with TN demonstrated that adhesion and migration were promoted by TN. The level of adhesion was generally less than that observed on other ECM molecules and it has been hypothesised that an intermediate level of adhesion facilitates invasion (Aznavorian *et al.*, 1992). The isoform of TN used in the functional assays was one whose size indicates that it contains the alternatively spliced region. Different isoforms of TN have been shown to possess different properties, as is discussed below.

The present study has indicated, by RT-PCR data that different isoforms of TN are expressed in malignant and benign/normal ovarian tissues. The existence of multiple TN isoforms, may in part explain the different and often contradictory results observed in studies of TN function. Differences in biological function between TN isoforms have been reported. Chiquet-Ehrismann *et al.* (1991) reported that the smaller isoform of TN preferentially bound to fibronectin, this is consistent with the finding that the smaller form of TN is better incorporated into the matrix. The smaller form of TN, found in normal and benign situations, is likely to be incorporated into the matrix close to its site of production resulting in focal immunostaining. Larger isoforms do not bind fibronectin and are not efficiently incorporated into the matrix, therefore they may diffuse from their site of production. This observation may partly explain the distribution pattern of TN observed in malignant and benign tumours.

The contrary functions of TN, with regards to cell adhesion, were first investigated by Spring *et al.* (1989), who utilised recombinant TN

fragments. They demonstrated that TN contained both adhesive (within the alternatively spliced region) and anti-adhesive (within the EGF-like repeats) sites. Subsequent studies have identified at least four non-overlapping sites on TN have been shown to interact with the cell surface (Prieto *et al.*, 1992).

The priorities of any future work would therefore be to define the protein expression of the different TN isoforms in ovarian tumours and cell lines, in order to elucidate further the role TN plays in ovarian cancer.

6.5 Summary

Work presented within this thesis has demonstrated that there is overexpression of TN in malignant ovarian tumours, however, the protein can still be observed in normal ovarian tissue. The cellular source of TN in these tumours is likely to be fibroblast cells, as indicated by the levels of TN expressed by these cell types in culture, and the similarity of their TN RNA expression pattern to malignant tumours. Whether the protein expressed in the malignant situation is a different isoform was not determined, however RT-PCR data suggested that malignant tumours were capable of producing RNA splice variants of a different molecular weight to those found in normal or benign situations. The possibility of utilising an anti-TN antibody to deliver radiation or cytotoxic compounds to ovarian tumours, would rely on this differential expression of different TN protein isoforms in normal and malignant ovarian tissue. The expression of different TN isoforms in the matrix may also determine the role of this protein in ovarian cancer.

Evidence from in vitro experiments demonstrated that the synthesis of TN is under paracrine control. TN was induced in fibroblasts by soluble factors. TGF- β_1 produced a large stimulation of TN secretion in fibroblasts. mRNA for this growth factor is expressed by ovarian carcinoma cells and its expression in tumours correlated with the degree of TN staining; these data implicate TGF- β_1 as an important regulator of

TN in ovarian cancer.

Investigation of the interaction of ovarian carcinoma cells with TN revealed that TN could promote the adhesion and migration of these cells, yet probably was not involved in altering the growth rate. This implies that TN may be involved in the invasion and metastasis of ovarian cancer.

Chapter 7 References

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